

RIBOSE NUCLEIC ACID and THE CELL NUCLEUS.

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GENERAL INTRODUCTION.

Nucleic acids were discovered in the latter half of last century, and this period may well be said to mark the beginning of the study of the chemistry of the cell nucleus. The work was initiated by Miescher in an investigation of the chemistry of nuclei isolated from pus cells: from such nuclei Miescher, in 1868, prepared a substance soluble in sodium carbonate and precipitated from there by acidification with acetic acid. This substance, which Miescher named 'Nuclein', was shown by him to give typical protein reactions and to contain greater amounts of phosphorus than any naturally occurring organic compound then known.

This work was carried out in Hoppe-Seyler's laboratory, but when submitted by Miescher for publication was withheld by the former while similar investigations were carried out by himself and his pupils. Simultaneously with the publication of Miescher's results (Miescher, 1871a) there thus appeared papers by Hoppe-Seyler and his school claiming the isolation of similar nucleins from yeast (Hoppe-Seyler, 1871), from the nuclear material of bird and reptile erythrocytes (Ploetz, 1871) and from a peptic digest of caesin (Lubaryn, 1871). Also a second paper was published by Miescher (1871b) claiming the presence of another nuclein in egg yolk. Thus, within three years of Miescher's original discovery in 1868 there were isolated the 'nucleins'



from five different sources - pus cell nuclei, yeast, nucleated erythrocytes, caesin and egg yolk; from all of which the nuclei were characterised as phosphorus containing organic acids soluble in alkali to give solutions precipitable by acid. NS

Miescher next turned his attention to salmon sperm, which he was fortunate in being able to obtain in large amounts from the Rhine fisheries at Basle where he was then working. In the course of a thorough chemical and biological investigation of this material he derived a suitable method for isolating the sperm heads, which he realised to be pure nuclear material, and from such sperm heads he (see Miescher, 1897) prepared two substances - a base, protamine, which he believed to be a simple substance, and an acid similar to the nuclein of pus cell nuclei but differing from it in being protein free. As is evident from Miescher's analytical results, the nuclein which he isolated from salmon sperm was the first member of this type of chemical compound to be obtained in a substantially, if not completely, pure state.

Further work was tedious owing to the lack of suitable methods for the isolation of the nucleic acids, as nuclein had been named by Altmann (1889), but in 1894 Kessel and Neumann derived a method for preparing nucleic acids from thymus glands which yielded the material used by Kessel in his classical investigation of nucleic acid chemistry.

Neumann (1899) later developed the method of preparation into one capable of giving good yields from all the common sources of nucleic acid except yeast - no reliable method became available for the preparation of Hoppe-Seyler's yeast nucleic acid until the early years of the present century (Kowlevsky, 1910). Using the preparative methods available Kossel (1891) showed the difference between the true nucleins - eg. from yeast or thymus, - which yielded purines on hydrolysis, and the pseudo-nucleins of egg yolk and caesin which, although containing phosphorus, yielded no purines on similar treatment. Thus he laid the foundations of modern nucleic acid chemistry. This <sup>means</sup> of characterising a true nucleic acid chemically left the way clear for the thorough chemical investigations which were being carried out in many different laboratories at that time.

By the second decade of this century, the chemistry of nucleic acids had developed sufficiently to allow the recognition of two quite distinct types (see Jones, 1914).

1. Animal nucleic acid - typified by Miescher's salmon sperm nuclein.
2. Plant nucleic acid - typified by Hoppe-Seyler's yeast nuclein.

The above nomenclature indicates sufficiently the distribution of the two types of nucleic acid <sup>which was then assumed.</sup>

By the time of the appearance of Levene's monograph (Levene and Bass, 1931) the

position regarding the chemistry of nucleic acids was much clarified: the salient features of their structure were known and the above two classes had been differentiated as Deoxyribose nucleic acid (Animal nucleic acid) and Ribose nucleic acid (Plant nucleic acid). At this time it was still generally assumed that deoxyribose was purely an animal product while ribose nucleic acid was confined to the plant kingdom, although Hammersten (1894) had many years previously isolated a yeast type of nucleic acid from the pancreas, which finding was confirmed and expanded by Jorpes (1928).

Other evidence was available, however, to show that the currently accepted distribution of the two acids was wrong. This was the powerful and important histochemical test of Feulgen and Rosenbeck (1924) for the detection of deoxyribose nucleic acid in histological preparations by virtue of its ability to give a pink colour with Schiff's reagent after acid hydrolysis (Feulgen, 1915). Using this test, these workers had shown the presence of deoxyribose nucleic acid in nuclei of both plant and animal cells. The importance of this work was not immediately recognised and it appeared to be disregarded to a considerable extent as it was not mentioned in Levene's otherwise comprehensive monograph. Feulgen and Rosenbeck were themselves unable to demonstrate the presence of deoxyribose nucleic acid in yeast but with the refinement of their

technique its occurrence there has been demonstrated by many workers, eg. Winge, (1935) and Delaporte, (1938). Following up this identification of deoxyribose nucleic acid in plant nuclei, Feulgen et. al. (1937) prepared the substance in question from the isolated nuclei of rye germ, so proving beyond all doubt the presence of deoxyribose nucleic acid in plant cells. It had therefore become obvious that the terms plant and animal nucleic acids were no longer valid, nor the implied distinction correct, and the idea arose of deoxyribose nucleic acid being a true nucleic acid, that is, a universal constituent of the cell<sup>nuclei</sup> of both plants and animals, as indicated by the work of Feulgen, while ribose nucleic acid was regarded as a purely cytoplasmic substance and therefore not a nucleic acid in the original sense of the word.

At about this stage in the study of nucleic acids there arose the conception which was to influence all later work on the more physiological aspects of nucleic acid chemistry: this was the concept of nucleoproteins, whether of the deoxyribose or ribose type, forming self duplicating systems. This idea was a direct result of the discovery by Stanley (1935) that Tobacco mosaic virus could be obtained as a pure nucleoprotein, a discovery which was soon followed by the isolation of several other plant and animal viruses, all of which were shown to be, or to contain, ribose or deoxyribose nucleoprotein. It was this concept of nucleoproteins forming the self duplicating system of viruses which made the



way easy for Caspersen's (1936,1939) claims that the chromosomes were composed of deoxyribose nucleoproteins, as the analogy between genes and viruses had long been stressed (Muller,1922).

In recent years the presence of ribose nucleic acid in animal tissues has been confirmed by many different workers using as many different techniques. Claude (1939,1941) was the first to produce definite evidence of its presence in the cytoplasm of animal cells: he isolated mitochondria and secretory granules from a variety of tissues by a process of differential centrifugation and showed them to contain ribose nucleic acid, both by analytical methods and by isolation. Previously Behrens (1938) had isolated ribose nucleic acid from the separated cytoplasm of rye germ but this work by Claude showed conclusively for the first time the widespread occurrence of ribose nucleic acid in the cytoplasmic granules of animal cells. Other evidence was seen forthcoming. Ribose nucleic acid was isolated from sheep liver (Davidson and Waymouth, 1944a) and from Calliphora larvae (Khouvine and Grégoire, 1944). It was shown to be present in a large number of tissues by estimation (Brachet, 1942a, Davidson and Waymouth, 1944b), by ultra-violet spectrophotometry (Caspersen et al. 1936-1942) and by histochemical methods (Brachet, 1942b). On the basis of such work it now seems likely that ribose nucleic acid is a universal



constituent of the cytoplasm of animal cells and so presumably of plant cells although evidence ~~for~~ the latter has been presented<sup>only</sup> in the case of rye germ ~~only~~ (Behrens, 1938), of yeast (Delaporte, 1938) and of root tip cells of SPINACIA (Caspersen and Schultz, 1940).

Nevertheless, ribose nucleic acid is not now considered to be solely a cytoplasmic constituent. On the contrary, much evidence, mainly of an indirect nature, has been advanced suggesting its presence in the nucleus, especially in the nucleolus. Caspersen and Schultz (1940) were the first to claim that the nucleolus contained ribose nucleic acid, basing their claims on the absorption maximum of the nucleolus at 260m $\mu$ , which showed the presence of nucleic acid, while the negative Feulgen staining reaction indicated the absence of deoxyribose nucleic acid. This claim was simultaneously and independently put forward by Brachet (1940) on the basis of his histochemical test, which was also used by Roskin (1945) with similar results. Davidson and Waymouth (1946) partly supported the claims that the nucleolus contained ribose nucleic acid using a combination of ultra-violet photography and of enzyme techniques but also suggested that the peripheral part of the nucleolus contained deoxyribose nucleic acid, as had previously been proposed by Seshachar (1942).

This claim that the nucleolus contains ribose nucleic acid has been widely accepted by cytologists and geneticists and many complex theories relating to gene action have been built up on the assumption of its truth, especially theories relating the nucleolus to heterochromatin and to protein synthesis. Caspersen and his co-workers in particular have developed a highly speculative theory of nucleolar function based on this assumption. These theories have been summarised (Caspersen 1947) - 'A certain part of the chromatin, we call it the nucleolus associated chromatin, secretes substances of a protein nature: there are very strong indications that they contain considerable amounts of diamino acids. These substances accumulate and form the main bulk of the large nucleolus. From the nucleolus they diffuse towards the nuclear membrane on the outside of which an intense production of ribose nucleic acid takes place. At the same time the amount of cytoplasmic protein increases.' Again Schultz (1947), after stating that both the nucleolus and the heterochromatin contain ribose nucleic acid and histones, remarks 'This fact lies at the base of the theory that the heterochromatic regions have to do with cytoplasmic synthesis in general by way of precursors derived from the nucleoli.' These two statements show the importance attached by cytologists to the assumed ribose nucleic acid of the nucleolus.

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The evidence for the occurrence of ribose nucleic acid therein, however, is far from satisfactory. Ultra-violet spectrophotometry can alone give little information but must be used in conjunction with some such histochemical test as that of Feulgen. The Possibility of locating the exact position of deoxy-ribose nucleic acid in the nucleus by means of this test has, however, been severely questioned (Stedman and Stedman, 1949). Other staining techniques, such as Brachet's histochemical test, are of doubtful value as will be discussed later.

A few determinations of the ribose nucleic acid content of isolated cell nuclei have been reported. The results of those are summarised in Table I. These results have, however, in the main, been obtained incidentally to some other project and in many cases are of doubtful value. This is due firstly to unexpected errors involved in the estimations based on the formation of furfural by the acid hydrolysis of ribose nucleic acid, and secondly to the use of impure nuclei. For instance, Schneider used the 'nuclear fraction' from rat liver with a total nucleic acid content of 9.4% as against approximately 27% in the preparations to be described later. The only values upon which any great reliance can be placed would appear to be those given by Vendrely (1948) and Davidson et al. (1949) and it should be noted that this present work was practically completed before the latter results were published.



Table 1.

Author.	Tissue.	Total N.A.	R.N.A.as % total N.A	R.N.A.as % nucleus.
Brachet, 1942a.	Frog intestine	28.7	9.8	-
Davidson and Waymouth, 1944b	Sheep embryo liver.	-	14	-
Euler and Hahn, 1946.	Calf thymus.	-	7	-
	Jensen sarcoma	-	8.1	-
Hammersten, 1946.	Fowl erythro- cytes.	-	1	-
Schneider, 1947.	Rat liver,	9.4	-	1.51
	kidney,	11.0	-	0.84
	hepatoma	11.9	-	1.42
Vendrely, 1948.	Ox liver,	-	4.4	-
	thymus.	-	6.9	-
Davidson et al. 1949.	Rabbit liver.	-	8.8	-
	Fowl erythro- cytes.	-	3.3	-

The present investigation was therefore undertaken with a view to determining the ribose nucleic acid content of nuclei isolated from as many different types of tissue as available using methods as free as possible from the defects mentioned above.



SECTION A.

Estimation of Ribose Nucleic Acid.

## INTRODUCTION.

Theoretically, nucleic acids may be estimated by any of the following methods:-

1. Estimation of nucleic acid phosphorus.
2. Estimation of nucleic acid purines or pyrimidines.
3. Estimation of nucleic acid sugar.

Of these methods (1) is the simplest but is open to criticism in that both ribose and deoxyribose nucleic acid contain phosphorus so that a satisfactory procedure must involve a quantitative separation of the two acids. The method of Schmidt and Thannhauser (1945) is of this type and depends on the alkaline hydrolysis of ribose nucleic acid to acid soluble products under conditions in which deoxyribose nucleic acid is unattacked. At first sight this method did not appear suitable for the present problem which was likely to involve the estimation of small amounts of ribose nucleic acid.

Method (2) was not considered further owing to the unreliability of the methods available for the separation and estimation of purines and pyrimidines. Such a method, however, had previously been used by Schmidt and Levene (1938).

The last possibility, the estimation of the sugar - i.e. ribose - seemed the most promising method as it was both sensitive and specific in that deoxyribose nucleic acid could not interfere. Many methods were available for the estimation of ribose nucleic acid by sugar determinations as for example

the various modifications of Bial's orcinol reaction (Dische and Schwartz, 1937; Mejbaum, 1939; Schneider, 1945) or the phloroglucinol reaction of Euler and Hahn (1946). The most suitable method appeared to be the acid hydrolysis of ribose nucleic acid to give furfural which could then be estimated by the well known colour reaction with aniline acetate.

Two such methods had recently been described - by Brachet (1942a) and by Davidson and Waymouth (1944b). The two methods differed greatly in detail, however. Brachet used strong sulphuric acid as the hydrolysing agent and carried out a simultaneous hydrolysis and steam distillation in the apparatus of Bailey (1937) before estimating the furfural in the distillate by its colour reaction with aniline, according to Hoffman (1927). Davidson et al. on the other hand, hydrolysed under the conditions of Reeves and Munro (1940) with hydrochloric acid in the presence of xylene: the furfural was estimated in the organic solvent, again using the method of Reeves and Munro. The method of Brachet appeared to be difficult to control accurately, while that of Davidson et al. suffered from the disadvantage of carrying out the furfural estimation in an organic solvent in which the colour developed is unstable, and which also takes up dark coloured materials formed by hydrolysis of any carbohydrates present. For this reason Reeves and Munro found it necessary to distill the xylene solution of furfural

carefully in vacuo when carbohydrates other than pentoses were present. The use of hydrochloric acid, however, appeared definitely advantageous as the experimental conditions are easily controlled. The method was known moreover, from the work of Reves and Munre, to give reliable results with free pentoses.

It was decided, therefore, to combine the methods of Brachet and of Davidson by hydrolysing with hydrochloric acid yet separating the furfural from other products by steam distillation after the hydrolysis was complete. A difficulty inherent in the method was the instability of the colour produced by the condensation of furfural with aniline acetate. This was partly reduced by the use of aqueous solutions and obviated entirely by the use of the reagent of Ree and Rice (1948) in which the colour is stabilised by development in the presence of excess thiourea as an anti-oxidant.

In the early stages of this work it appeared that no separation of the nucleic acids such as that carried out by Davidson and Waymouth (1944b) or by Euler and Hahn (1946) would be necessary prior to estimation owing to the relative simplicity of the material under investigation - isolated cell nuclei consisting, for all practical purposes, of deoxyribose nucleic acid (30%-40%), basic protein (20%-30%) and chromosomin (30%-50%). In the course of the preparation of the cell nuclei (see section B) any pentose containing compound of low



molecular weight would be eliminated, so interference from that source was unlikely. As will be described later, however, a preliminary separation of ribose nucleic acid from deoxyribose nucleic acid was found to be necessary because of an unsuspected effect of the latter on the estimation.

In the course of the work an examination of Brachet's method was carried out.



## EXPERIMENTAL.

### ESTIMATION OF FREE FURFURAL.

As a preliminary to using the proposed method for the determination of ribose nucleic acid, the suitability of the colour reagent for the estimation of furfural itself was investigated. The following reagents were used.

FURFURAL. B.D.H. 'specially purified' furfural was freshly distilled in vacuo in an all-glass apparatus and the colourless distillate dried over  $\text{Na}_2\text{SO}_4$ . Solutions in water of the requisite concentration were prepared from this dry material.

ANILINE. This was distilled in 25ml. portions from zinc dust and the distillate kept at 0°C in a dark bottle.

ACETIC ACID. Glacial acetic acid 'Analar' quality was purified by refluxing for three hours with 2% of chromic oxide and was then distilled.

THIOUREA. This was twice recrystallised from water.

ANILINE ACETATE REAGENT. 2ml. aniline were dissolved in 100ml. glacial acetic acid previously saturated with thiourea. Roe and Rice stated that the use of aniline acetate sometimes gave rise to brown colours in the estimation of pentoses in tissue extracts and therefore recommended the use of p-bromoaniline in place of the simple base. In the present investigation it was found that the yellow-brown colour was due to the use of insufficiently purified acetic acid and could be completely avoided by

purifying it as described above.

METHOD. A 5ml. aliquot of the solution containing furfural, in a concentration of 5-15 $\mu$ g/ml, was treated with 30ml. of the aniline acetate reagent in a loosely stoppered tube and the mixture heated at  $70^{\circ}\text{C} \pm 2^{\circ}\text{C}$  for 15 minutes. It was then cooled under the tap and incubated at  $37^{\circ}\text{C}$  in the dark for 45 minutes, cooled to room temperature, stood in the dark for 15 minutes and its extinction measured in the Spekker Absorptiometer using a 'Spectrum Green' filter (520 m $\mu$ ) and a 4cm. light path.

In very cold weather it was advisable to warm the Spekker cells slightly to avoid cooling the solutions too strongly, otherwise thiourea tended to crystallise out during the estimation.

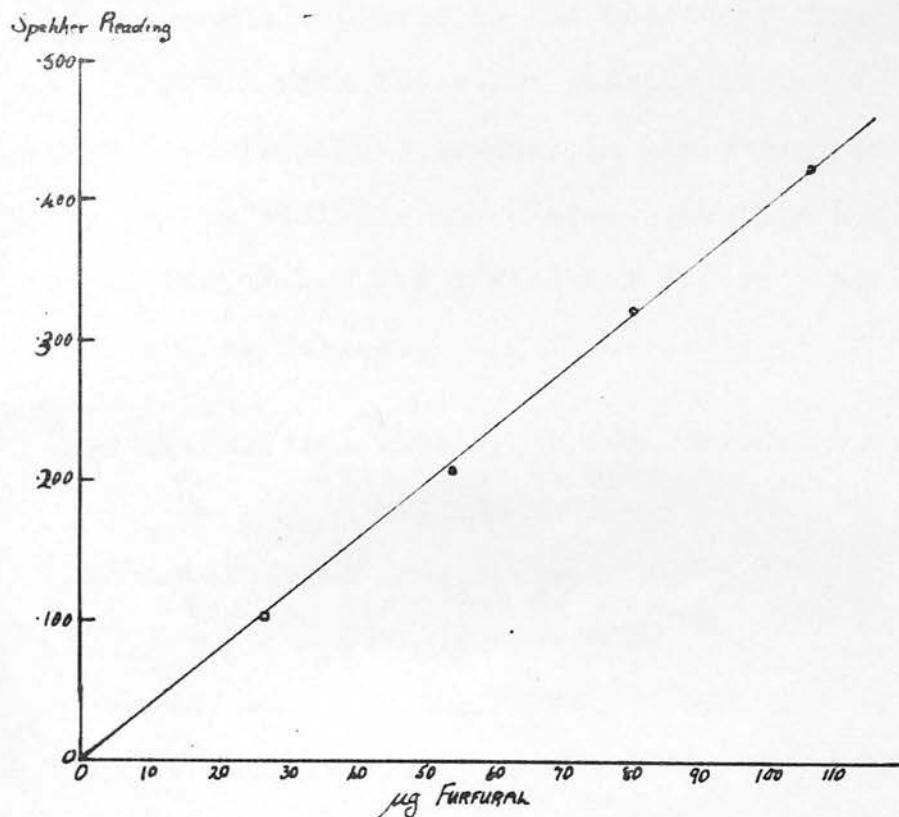
The results obtained with furfural are shown in Figure 1, which indicates the suitability of the method. It was accordingly used in subsequent experiments for the determination of furfural in steam distillates.

It was sometimes advantageous when estimating more concentrated solutions of furfural (25-100  $\mu$ g./ml.) to use a 1cm. light path in the Spekker Absorptiometer. In such circumstances a 2ml. aliquot of the solution was treated with 10ml. aniline acetate reagent in the above manner, the results being read from the appropriate calibration curve.

# -FURFURAL CALIBRATION CURVES-

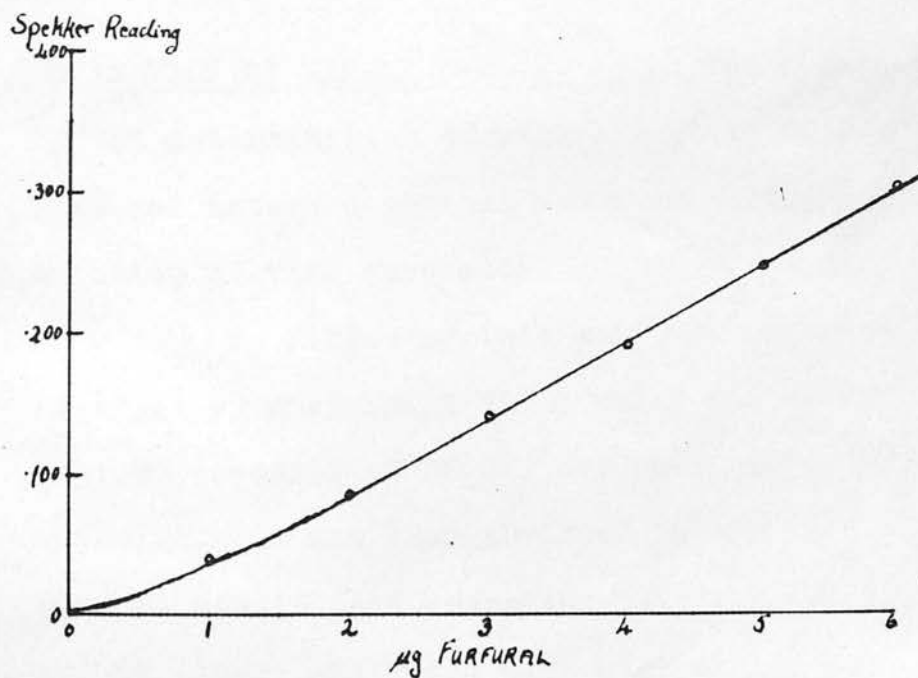
## ① Method of Roe and Rice

Fig. 1



## ② Method of Brachet

Fig. 2



EFFICIENCY OF STEAM DISTILLATION. In the estimation of ribose nucleic acid it was necessary to separate the furfural from the other hydrolysis products by steam distillation. Brachet, in his method, collected only 15ml. distillate and claimed quantitative recovery of furfural. The efficiency of the distillation was shown as follows.

0.114mg. Furfural made up to 50ml. water-Spekker	0.072
"                  distilled to 50ml.                  "	0.022
"                  distilled to 25ml.                  "	0.144
0.228mg. Furfural made up to 50ml.                  "	0.145
"                  distilled to 50ml.                  "	0.145
"                  distilled to 25ml.                  "	0.290

It was obvious from the above results that furfural could be quantitatively recovered by steam distillation of only 25ml. The distillation was carried out at such a rate that 25ml. slightly warm distillate was collected in 3-4 minutes.

ESTIMATION OF RIBOSE NUCLEIC ACID. The applicability to the determination of ribose nucleic acid of the furfural method described above was examined on a solution of this substance.

Ribose nucleic acid (B.D.H.) was purified by suspending it in water and adding the minimum quantity of  $\text{Na}_2\text{CO}_3$  necessary to effect solution. It was then purified by the addition of glacial acetic acid according to the method of Kunitz (1940) and dried with alcohol and ether.

Analyses of this specimen after drying over  $\text{P}_2\text{O}_5$  in high vacuum gave the following results



N - 16.45%

P - 9.09%

N/P - 1.70.

From this material, dried for three days as in the above analyses, standard solutions of suitable concentrations were prepared. These were treated as follows.

A 3ml. aliquot of a suitable solution was hydrolysed under reflux with 3ml. of exactly 7N HCl for  $2\frac{1}{2}$  hours in a boiling water bath. The solution was cooled, the condenser ringed, the hydrolysate made just alkaline to methyl orange with 6N NaOH and immediately distilled in an all-glass apparatus under the conditions described above, the volume of the distillate being adjusted to exactly 25ml. A suitable aliquot of the distillate, normally 5ml, was taken for furfural estimation as described on p.16.

The results are shown in Figure 3, in which the extinction is plotted against the weight of ribose nucleic acid present in the 3ml. aliquot employed. It is evident from this figure that the extinction is directly proportional to the weight of ribose nucleic acid present and it is therefore concluded that under the <sup>conditions</sup> ~~circumstances~~ chosen the method is suitable for the determination of small quantities of pure ribose nucleic acid in solution.

EFFECT OF DEOXYRIBOSE NUCLEIC ACID. As the object in view was the application of this method to the determination of ribose nucleic acid in isolated cell nuclei, the possibility of recovering added ribose nucleic acid from such nuclei was examined. For this



this purpose a solution containing 2.2mg. ribose nucleic acid was added to 100mg. dyr nuclei from calf ~~thymus~~ gland and the mixture hydrolysed and steam distilled under the conditions described above. The experiment was carried out in duplicate and in each case gave a yield of 0.93mg. ribose nucleic acid. The recovery of the nucleic acid was thus only about 40% of the added material.

The most likely explanation of this discrepancy was that some material present in the nuclei was either inhibiting the production of furfural, or combining with it once it was formed, and preventing its estimation. Proteins or their hydrolysis products seemed a likely source of error, but the addition of histone or <sup>of</sup> ~~ether~~ amino acids to the ribose nucleic acid estimations had no effect. Deoxyribose nucleic acid, however, was shown to have a definite effect on the estimation of ribose nucleic acid (R.N.A.). 1.59mg. R.N.A. estimated in the presence of 30mg. deoxyribose nucleic acid (D.R.N.A.) gave a recovery of only 0.83mg. R.N.A. A slight inhibition was also noted on adding chromosomin to an estimation. This was small enough, however, to be due entirely to traces of D.R.N.A., amounting to not more than 1.8%, in the specimen of chromosomin used.

It was obvious, therefore, that the inhibitory effect of cell nuclei in the estimation of ribose nucleic acid was due to the presence of deoxyribose nucleic acid, or more probably, of one of

its hydrolysis products. This latter possibility was further supported by the following experiment.

Ribose nucleic acid and deoxyribose nucleic acid were hydrolysed separately and steam distilled in the usual manner, sufficient deoxyribose nucleic acid being used to cause a 60% inhibition of furfural production when the two acids were hydrolysed together. On mixing the distillates and estimating the furfural only a small diminution (12%) of the apparent furfural content was noted -

Equal volumes of R.N.A.hydrolysate and water --  
O.75mg.R.N.A.

Equal volumes of R.N.A.hydrolysate and D.R.N.A.  
hydrolysate - 0.66mg.R.N.A.

This suggested that the inhibitory action of deoxyribose nucleic acid occurred mainly during the hydrolysis, probably by some volatile intermediate of the deoxyribose nucleic acid hydrolysis combining with the furfural and rendering it non-volatile, or in some way unreactive with the aniline acetate reagent.

Stacey et al.(1946) suggested that  $\omega$ -hydroxy-laevulinic aldehyde was an intermediary in the hydrolysis of deoxyribose nucleic acid. It seemed likely, therefore, that such a substance might well combine with furfural and so render it unreactive. Methoxy-laevulinic aldehyde dimethyl acetal was prepared by methanolic HCl hydrolysis of furfuryl alcohol, according to Pummerer (1935). On the addition of this acetal to ribose nucleic acid an

inhibition of furfural production on hydrolysis was noted - 1.47mg.R.N.A. taken ; 0.59mg.R.N.A. recovered in the presence of 150mg. acetal. This indicated that the free hydroxylaevulinic aldehyde, which is formed on the acid hydrolysis of the acetal, could interfere with the estimation of ribose nucleic acid, and so gave a probable explanation of the previously unrecognised inhibitory effect of deoxyribose nucleic acid on the formation of furfural from ribose nucleic acid.

The mechanism of the inhibitory action of deoxyribose nucleic acid on the furfural formation, which was subsidiary to the main problem of this investigation, was not examined further. The fact that deoxyribose nucleic acid produced such an effect was sufficient to render the proposed method for the estimation of ribose nucleic acid in cell nuclei inapplicable in its simple form.

BRACHET'S METHOD.

In view of the difficulties encountered in the application of the simple method to the determination of ribose nucleic acid in complex mixtures, it was decided to investigate Brachet's procedure (1942) for this purpose. According to Brachet's results, this gives remarkably accurate and consistent results when applied to pure ribose nucleic acid, the yield of furfural corresponding almost exactly to its total content of ribose. This is contrary to expectation, for, according to current views, only 50% of the ribose should be liberated, and therefore made available for the production of furfural during hydrolysis. It was thought that the drastic hydrolytic procedure used by Brachet might be responsible for this peculiarity in his results. There also seemed to be a possibility that the removal of furfural continuously, as it was formed in the hydrolysis mixture, might prevent the destructive effect of deoxyribose nucleic acid.

FURFURAL ESTIMATION. With pure solutions of furfural, the development of the colour with the aniline acetate reagent used by Brachet was satisfactory. Thus 6ml. of a solution of furfural were treated with 0.5ml. aniline and 4ml. glacial acetic acid. The development of colour was followed in the Spekker Absorptiometer using the 'Spectrum Green' filter. It developed rapidly, reached a maximum at 25 minutes and remained constant for 10 minutes before falling



slowly and linearly.

Figure 2 shows a calibration curve prepared from a standard furfural solution using the above method and reading the colour intensity 30 minutes after mixing. The method appears to be satisfactory and is much more sensitive than the method previously described, estimating between  $1\mu\text{g.}$  and  $10\mu\text{g.}$  furfural in 6ml. It is of interest that the relationship between <sup>the amount of furfural and the</sup> colour intensity (Fig.2) is not strictly linear, as it is in the method of Roe and Rice (Fig.1). An unsatisfactory point in the estimation, however, is that the solutions are slightly turbid, presumably due to an excess of aniline, a fact which cannot enhance the accuracy of the method.

ESTIMATION OF RIBOSE NUCLEIC ACID. In this the hydrolysis was carried out exactly as described by Brachet, but the furfural was estimated by the method described in the previous section rather than by Brachet's own method.

A convenient amount of ribose nucleic acid was hydrolysed with 3ml.  $\text{H}_2\text{SO}_4$ , 35%, saturated with  $\text{K}_2\text{SO}_4$  and containing  $\text{ZnSO}_4$ , 8%. Simultaneous hydrolysis and steam distillation was carried on for 25-30 minutes, the hydrolysate being allowed to concentrate during the last 10 minutes so that  $\text{K}_2\text{SO}_4$  crystallised out. 25ml. distillate, which was strongly acid to litmus, was collected and the furfural estimated as usual. Accurate control and duplication of the conditions was very difficult. The

following are typical yields of furfural from ribose nucleic acid -

(1) 15.7%                      (2) 14.2%                      (3) 10.5%

The theoretical yield, assuming hydrolysis of both purine and pyrimidine nucleotides, is 29.4%, or 14.7% for hydrolysis of the purine nucleotides only. The above are apparently normal yields of furfural but several very low values, in which the yield was only about 5%, were obtained under what appeared to be identical hydrolysis conditions. The value of 15.7% is the maximum yield of furfural obtained in the present investigation.

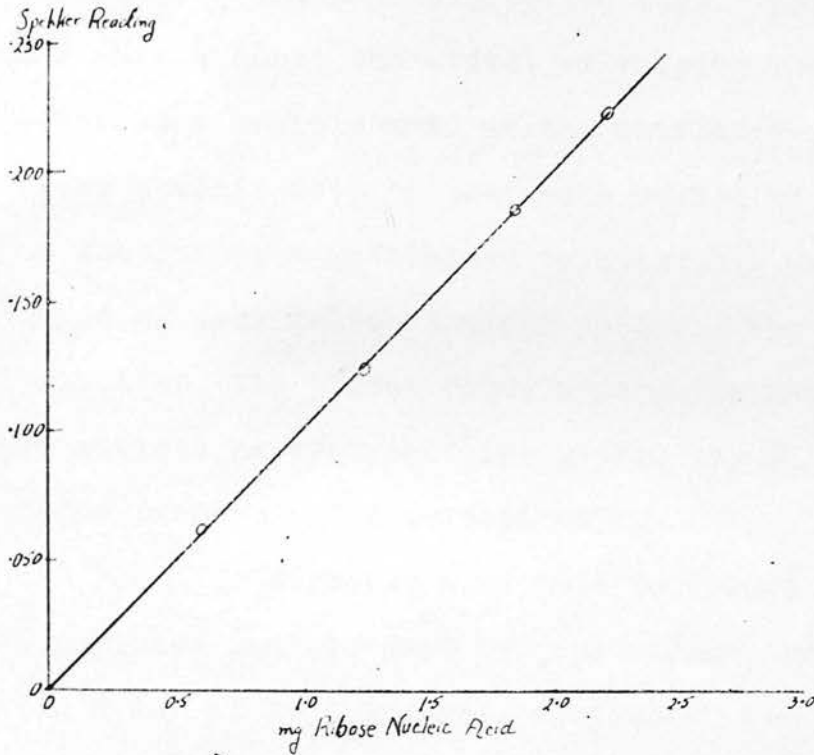
It was apparent that the method as described by Brachet could not give reliable results. Before discarding it, however, a modified method was investigated.

A 1ml. aliquot of the solution under investigation was simultaneously hydrolysed and steam distilled with 3ml.  $\text{H}_2\text{SO}_4$  saturated with  $\text{K}_2\text{SO}_4$ . Hydrolysis was continued for about 40 minutes, the temperature of the reaction mixture being kept constant at 160°C in an oil bath; the rate of steam<sup>flow</sup> was adjusted so that the volume of the hydrolysate remained constant. 50ml. distillate was collected and a suitable aliquot taken for the furfural estimation in the usual method.

Figure 5 shows a calibration curve prepared from ribose nucleic acid by the above

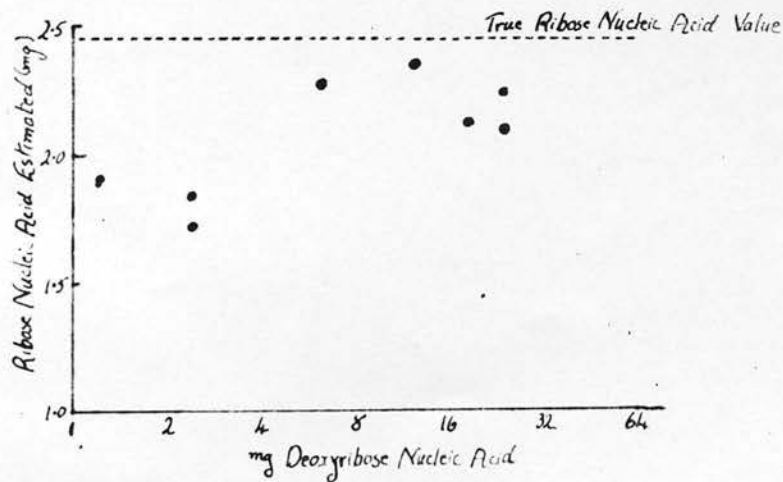
~ RIBOSE NUCLEIC ACID CALIBRATION ~  
Modified Brachet method

Fig 5



~ EFFECT OF DEOXYRIBOSE NUCLEIC ACID ON ESTIMATION ~  
(Modified Brachet method)

Fig 6



method.

The method was apparently quite successful for pure ribose nucleic acid but once again <sup>deoxy</sup>/ribose nucleic acid had an inhibitory effect. This is shown in Figure 6 where the effect of varying ~~the~~ amounts of deoxyribose nucleic acid on the estimation of 2.45mg. ribose nucleic acid by the above method is indicated. The results show no obvious relationship between the amount of deoxyribose nucleic acid and the degree of inhibition, the effect being apparently random. It was obvious, however, that the method was of no value in the problem under investigation.

Attempts were made to destroy the deoxyribose nucleic acid by preliminary incubation with  $H_2SO_4$  at 37°C before hydrolysis. The method proved to be unreliable although some degree of success was obtained.



FINAL METHOD.

The failure of Brachet's method to prevent the destructive action of deoxyribose nucleic acid on furfural appeared to leave only one solution to the problem, namely the complete removal of deoxyribose nucleic acid from the material under investigation. An apparently suitable method had been described by Schmidt and Thannhauser (1945). This depends on the difference in stability of the two acids to warm alkali. If material such as dried tissue containing both nucleic acids is digested with warm alkali the ribose nucleic acid is hydrolysed completely into products not precipitated by the addition of hydrochloric acid ~~and~~ trichloroacetic acid whereas deoxyribose nucleic acid remains unchanged, or at least yields products which are so precipitated. In using this procedure it was considered advisable to omit trichloroacetic acid from the precipitating agent in view of the possible destructive action it might exert on the furfural formed. The complete procedure developed is as follows -

A suitable quantity of the substance containing ribose nucleic acid was digested for 18 hours at 37°C with 2ml. N KOH in a graduated centrifuge tube, the whole of the material passing into solution during the first two hours. The resulting clear solution was acidified with 0.4ml. 6N HCl which precipitated any deoxyribose nucleic acid and protein present as a sticky mass. After standing for 30

minutes at 0°C the volume was made up to 4ml. with 0.1N HCl and the dense precipitate removed by centrifuging for 10 minutes at 3000 r.p.m. The clear supernatant was decanted off from the compact precipitate and a suitable aliquot, normally 2ml, taken for the estimation of ribose nucleic acid as described on p.17

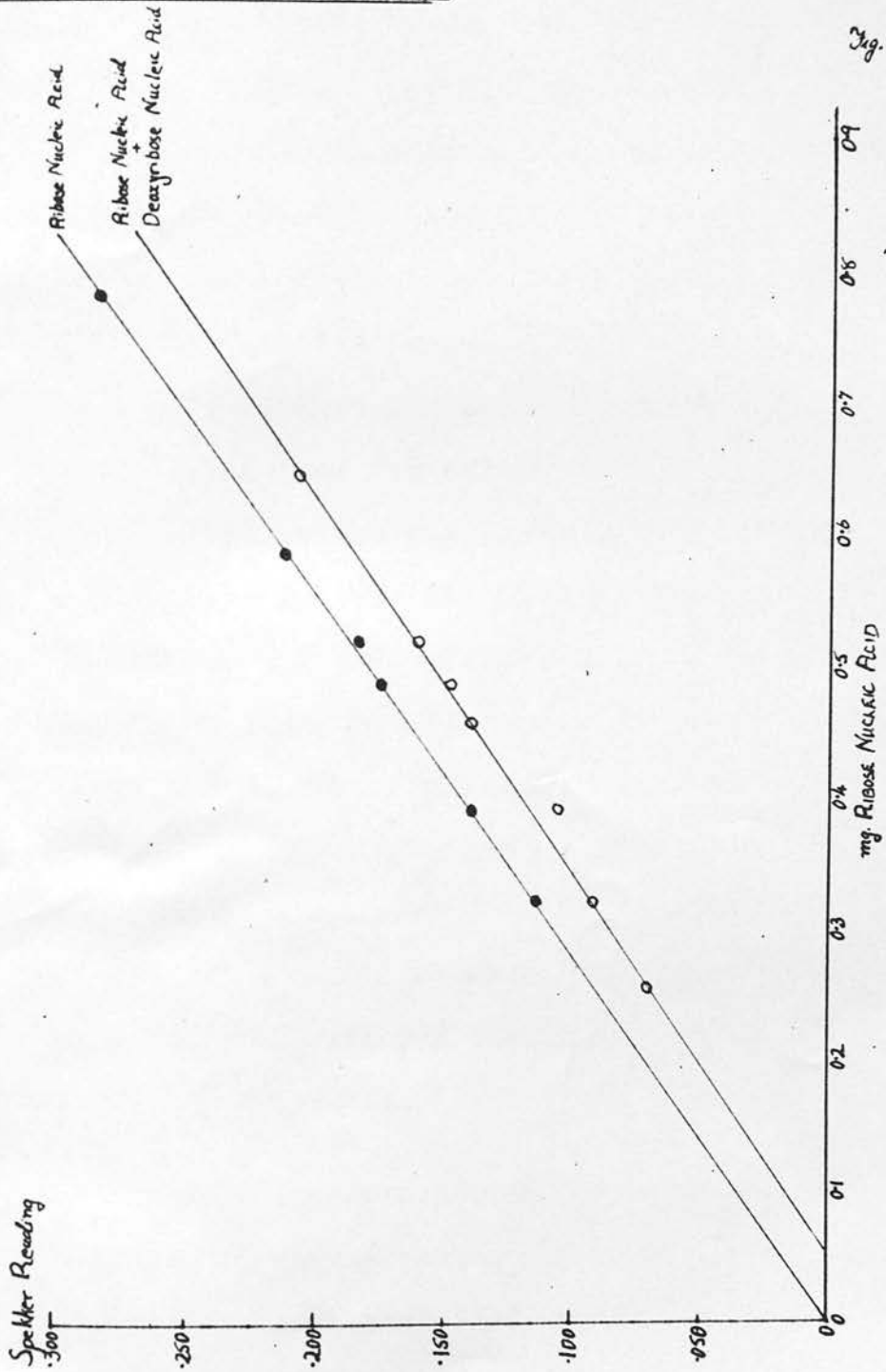
Figure 7 shows a calibration curve plotted from results obtained by this method with ribose nucleic acid alone. In the presence of a relatively large excess <sup>of deoxyribose nucleic acid,</sup> (about 50mg) a small but constant inhibition of furfural production occurred, as shown by the lower curve in Figure 7.

It should be mentioned that the deoxyribose nucleic acid used in the preparation of the above curve was obtained from herring sperm by Neumann's method, as described by Jones (1914). It was free from ribose nucleic acid but contained traces of protein.

The slight lowering of the ribose nucleic acid curve in the presence of deoxyribose nucleic acid suggested that the separation of the two acids was not complete. The presence of deoxyribose nucleic acid in the final centrifugates used in the estimation was, in fact, confirmed by the use of Dische's test with diphenylamine. In each case a faint but definite blue colour of approximately the same intensity was produced. This indicated that the incomplete precipitation of the deoxyribose nucleic acid was due to its slight solubility in the

- RIBOSE NUCLEIC ACID CALIBRATION -

Fig. 7



medium used. A similar conclusion can be drawn more convincingly from the calibration curves in Figure 7. From these results it would appear that the method described above, involving the removal of deoxyribose nucleic acid, could be used for the determination of ribose nucleic acid in cell nuclei, provided that allowance was made for the destructive effect on the furfural of the small but constant amount of deoxyribose nucleic acid remaining in the centrifugate - that is, providing that the lower calibration curve in Figure 7 were used for calculating the results. When, however, the method was actually applied to cell nuclei it was found that the deoxyribose nucleic acid content of the latter differed from the Neumann preparation used in the above experiments in that after digestion of the nuclei with alkali it was so completely precipitated from solution on acidification that the supernatant failed to give a trace of colour with Dische's reagent. The upper of the two calibration curves was therefore employed in calculating the results.

It appeared advisable to check the completeness of the hydrolysis of ribose nucleic acid under the above conditions, as Reeves and Munro (1940) obtained 100% conversion of xylose to furfural in boiling 3.5N HCl only after 2.5 hours. Figure 4, however, shows that the hydrolysis of ribose nucleic acid to furfural is complete in 2 hours, no further production of furfural occurring even after



3 hours. This difference between free xylose and ribose nucleic acid is probably due to the liberation of the ribose in an unstable form which is more rapidly converted into furfural.

The method described above therefore seemed suitable for the estimation of small amounts of ribose nucleic acid in cell nuclei ; recovery experiments were performed and it was found that 91%-93% of added ribose nucleic acid could be recovered from isolated nuclei using this procedure, which was therefore directly applicable to the problem under investigation.

### INTERFERING SUBSTANCES.

One possible defect in the method just described for the determination of ribose nucleic acid in tissue preparations is its lack of specificity for any substance yielding a pentose on hydrolysis must theoretically be capable of giving rise to furfural under the conditions employed. Compounds of this type occurring in the animal body are, however, rare. Apart from traces of pentoses, they seem to be represented chiefly by the co-enzymes and the prosthetic groups of certain of the yellow enzymes. It is very improbable that these occur in more than trace amounts in the nucleus. Nevertheless it cannot be excluded that a portion of the furfural yielded by cell nuclei on hydrolysis finds its origin in sources other than ribose nucleic acid.

Other compounds, including a variety of glucuronic acid derivatives, and, according to Brachet (1942), glycogen yield furfural on acid hydrolysis. It therefore seemed desirable to determine the yield of furfural from such compounds. The results obtained are collected in Table 2. As indicated, some of the estimations were made without, and others with, preliminary incubation with alkali. The results are expressed in two ways, firstly as yield of furfural as a percentage of the theoretical, and secondly as the ribose nucleic acid (R.N.A.) equivalent, which is defined as the number of milligrams of the substance producing the same amount of furfural as 1mg.

ribose nucleic acid. The R.N.A.equivalent is thus a measure of the interference brought about by the substance in question, the higher the value the less interference will be caused.

Table 2.

Substance investigated.	Without alkali treatment.		With alkali treatment.	
	Furfural yield.	R.N.A. equiv.	Furfural yield.	R.N.A. equiv.
Deoxyribose 1.	-	-	-	175
nucleic acid. 2.	0	-	0	-
Glycogen.	-	570	0	-
Heparin.	-	4.6	0	-
<u>d</u> -Glucurone.	20%	0.76	0	-
Phenol- $\beta$ - <u>d</u> -glucuronide.	17%	1.4	13%	2.6
<u>l</u> -Menthol- $\beta$ - <u>d</u> -glucuronide.	34%	2.0	-	-

The values indicated above show that under the experimental conditions adopted only the glucuronic acid derivatives, and of course co-enzymes, are likely to cause any serious interference.

Deoxyribose nucleic acid (1), which was prepared from thymus glands by Gulland's method, (see Gulland et al. 1947), gave a small yield of furfural. This was almost certainly due to contamination with ribose nucleic acid. Deoxyribose nucleic acid (2), a Neumann preparation from isolated herring sperm heads, gave, on the other hand, no furfural.

## DISCUSSION.

The method described ~~for~~ would appear to provide a satisfactory estimation for ribose nucleic acid in the presence of proteins and of deoxy-ribose nucleic acid. For pure ribose nucleic acid the method has an accuracy of not less than  $\pm 3\%$ , but in the presence of deoxyribose nucleic acid under the conditions existing in cell nuclei the figure falls to about  $\pm 7\%$ . Some of this loss of accuracy is doubtless due to the nature of the precipitate obtained on acidification of the alkaline digest, and this loss could be avoided to some extent by redissolving and reprecipitating this precipitate.

It is usually assumed that the moderately prolonged treatment with hot <sup>acid</sup>~~alkali~~ necessary to produce furfural from pentose-containing compounds causes an appreciable destruction of this substance. Reeves and Munro (1940), for example, claimed that this destruction could be completely obviated by carrying out the hydrolysis in the presence of xylene. The solvent was supposed to trap the furfural as fast as it was formed and to protect it from destruction in the aqueous phase. Since, however, the partition co-efficient of furfural between 3.5N HCl and xylene at 100°C is, according to Reeves and Monro, 0.39 it seems clear that the protective action of xylene can by no means be complete for an appreciable amount of the furfural formed at any point in the experiment would be present



in the acid phase and hence be susceptible to destruction. Nevertheless Reeves and Monro succeeded in obtaining theoretical yields of furfural from xylose, a result which appears to contradict the view that some destruction normally occurs during the hot acid treatment. The action of HCl on furfural under the conditions used (p.17) for the estimation of ribose nucleic acid was therefore examined. An aliquot of a standard solution of furfural was heated on a boiling water bath with 3.5N HCl for various periods of time, the contents of the flask steam distilled in the usual manner and the furfural contents of the distillate determined by comparison with the original standard solution. During the first two hours there was an apparent destruction of furfural amounting to 13.5% but the same result was obtained if the heating was prolonged up to 3.5 hours. It was clear that if 13.5% was destroyed in the first two hours this destruction should have continued during the subsequent 1.5 hours. That it did not do so is difficult to explain except on the basis that the furfural used was contaminated with other material. Furfural is notoriously difficult to purify and it is conceivable that an impurity was present in it which, during the first 2 hours of treatment with HCl, combined with and so caused the loss of a portion of the furfural.

The possible destruction of furfural during hydrolysis of ribose nucleic acid is, however,

without influence on the method for the estimation of the latter used in this investigation. The results are calculated from the upper curve in Fig.7 which shows that under the conditions employed the amount of chromogenic material, presumably furfural, produced from ribose nucleic acid is directly proportional to the weight of the latter substance.

#### EFFECT OF DEOXYRIBOSE NUCLEIC ACID.

The inhibition of the production of furfural from ribose nucleic acid by deoxyribose nucleic acid, or more probably the combination of the furfural with some deoxyribose nucleic acid hydrolysis product, is of great importance and does not appear to have been noted previously. Time was not available for a complete investigation of the effect but the experiments described in the experimental section suggest that it may be due to the combination of the furfural with some substance of the nature of hydroxylaevulinic aldehyde to give a non-volatile complex. Although the addition of methoxylaevulinic aldehyde dimethyl acetal to a ribose nucleic acid estimation gave an effect similar to that caused by the addition of deoxyribose nucleic acid, it is important to note that the acetal, or the parent aldehyde, has not been shown to be formed during the hydrolysis of deoxyribose nucleic acid. Stacey et al. (1946) showed that methanolic HCl hydrolysis of deoxyribose gave the acetal only under very vigorous conditions; because this acetal gave strongly positive Dische

and Feulgen reactions, they claimed that the parent aldehyde was formed in the normal hydrolysis of deoxyribose nucleic acid. The conditions employed by Stacey, however, were so different from those used in the normal hydrolysis of deoxyribose nucleic acid - 0.1% absolute methanolic HCl as against 12% aqueous HCl - that it is very difficult to attach much significance to these claims. Pummerer et al. (1935) <sup>claimed,</sup> however, that hydroxylaevulinic aldehyde was an intermediary in the formation of laevulinic acid from hexoses and as this acid is also formed <sup>by</sup> the HCl hydrolysis of deoxyribose nucleic acid, Stacey's claims may be not entirely groundless.

To whatever the effect if due, it is obviously of considerable practical importance. Table 3, columns 1 and 2, show the results of estimations on nuclei before and after separation of the two acids, while column 3 shows the yield before separation expressed as a percentage of the correct value. The magnitude of the effect is obviously considerable and shows the needs of careful interpretation of any results of ribose nucleic acid estimations by furfural production in methods not involving at least a partial separation of the two acids.

Table 3.

Material.	Before separatn.	After separatn.	% Yield.
Ox liver nuclei, 2/48	0.68%	1.1%	62
3/48	0.62%	1.0%	62
Ox thymus nuclei 10/48	0.34%	0.83%	41
3/48	0.58%	1.3%	45
Ox spleen nuclei 11/46.	0.49%	0.70%	70
10/47.	0.64%	1.1%	58

It is important to note that in the material under investigation the R.N.A./D.R.N.A. ratio is small, of the order of 0.05, while in whole tissue the ratio is much larger, often being greater than unity. Under such conditions, as in some of the estimations reported by Davidson (1947a), the effect may not be so important although Figure 6, showing the effect of varying the amount of deoxyribose nucleic acid on a ribose nucleic acid estimation by the modified Brachet method, suggests that the inhibition is significant over a wide range of values for the ratio R.N.A./D.R.N.A.

With regard to other furfural-yielding substances, apart from co-enzymes or mucoproteins, no substance is likely to be present in sufficient amount to cause interference. Glucuronides are unlikely to be present to sufficient concentration and glycogen could only interfere if present in impossibly large amounts. There is no reason to believe that deoxyribose nucleic acid could give rise to furfural, as is indicated by the experiments with herring sperm



nucleic acid, prepared by Neumann's method. The production of furfural from a specimen of thymus nucleic acid, prepared by Gulland's method is doubtless due to the presence therein of traces of ribose nucleic acid - as the material is prepared from whole glands contamination with cytoplasmic ribose nucleic acid is not at all unlikely.

#### BRACHET'S METHOD.

As is indicated in the experimental section, Brachet's method did not yield satisfactory results ; one of the most important factors is no doubt the difficulty of controlling exactly the conditions, even in consecutive determinations, as in the more readily controlled modified method ~~and~~ the duplication of results was much easier. Another factor is the pronounced acidity of the distillate. This makes the colorimetric estimation of furfural very unreliable, as was stressed by Hoffman (1927) who took extreme precautions to ensure the neutrality of the distillate.

Even allowing for the lack of precision of the method, no explanation can be offered for the great discrepancies in the yield of furfural from ribose nucleic acid as reported by Brachet, and the values given in the previous section. Brachet claimed that under his conditions both purine and pyrimidine nucleotides were hydrolysed, giving 29.4% yield of furfural. Carrying out the determinations exactly

as reported, the maximum yield obtained in this work was 15.7% : that is, apparently only the purine nucleotides were yielding furfural, as ~~is~~ known to be the case with 3.5N HCl hydrolysis. No explanation can be given for this discrepancy.

The modified method described would appear to be a considerable improvement on the older method, in that concordant results are readily obtained. As might be expected, the yield of furfural is again 14% - 15%. No attempt was made to develop the method as it is very sensitive to the presence of deoxyribose nucleic acid, as shown in Fig. 6, and possesses no advantages over the more readily controlled hydrochloric acid method already described.

SECTION B.

Ribose Nucleic Acid in Cell Nuclei.

## INTRODUCTION.

As pointed out in the General Introduction, few estimations of ribose nucleic acid in isolated cell nuclei have been reported, the available figures being given in Table 1. Owing to the general acceptance of the view, based mainly on cytological evidence, that ribose nucleic acid occurs in the nucleolus it appeared important to determine as accurately as possible the content of that substance in the nuclei isolated from as many different tissues as were available.

The present section is therefore concerned with the isolation of normal cell nuclei from various sources and the determination of their ribose nucleic acid content by the method described in the previous section. An extension of the work to regenerating cells is described in the following section.



## ISOLATION OF NUCLEI.

The methods used for the isolation of cell nuclei depend on their nature and mode of occurrence. Such methods can conveniently be divided into three groups according to the source of the desired nuclei in (1) fish sperm, (2) nucleated erythrocytes and (3) glandular or similar tissue. Each method will be described separately.

## FISH SPERM HEADS.

The method employed is essentially that described by Miescher (1897) for salmon sperm. Fish spermatozoa contain little cytoplasm : they consist mainly of a head, which is almost entirely formed of nuclear material, and a tail of cytoplasmic origin. In the fish this tail is very fine and easily removed but in mammals, on the other hand, the tail is a more massive structure and more resistant to damage (cf. Zittle and Zitin, 1942) so that it is doubtful if the procedure used for the isolation of fish sperm heads would be applicable without modification to the preparation of mammalian material.

In the present work sperm heads from the salmon, herring and cod have been prepared by an almost uniform procedure. The testis, obtained during the spawning season, and so containing ripe sperm, are disintegrated by mincing ; the resulting pulp is suspended in several times its own volume of water and stirred vigorously with a mechanical stirrer. The suspension is then strained firstly, if necessary, through a relatively coarse strainer and finally

through four layers of muslin. In this condition the spermatozoa cannot be readily centrifuged down but by making the suspension just acid to litmus with dilute acetic acid and allowing it to stand for some time, sedimentation takes place readily. The sperm heads, indeed, show signs of sedimenting without centrifuging.

At what stage the tails are lost is not certain. Mischer was of the opinion that the acetic acid actually dissolved them. To the author it seems more probable that they are detached during the mincing and stirring. In any case, the sediment of sperm heads is still contaminated with impurity, presumably in part consisting of, or derived from, the tails. This is removed by fractionally centrifuging the material. The sperm heads are resuspended in water maintained faintly acid to litmus with acetic acid, and the suspension centrifuged at the lowest requisite speed. The supernatant, which is faintly turbid, is rejected and the process repeated on the sperm heads until, after centrifuging, the supernatant is perfectly clear.

Photomicrographs of salmon sperm heads as they appear at this stage of preparation, after staining with methylene blue, are shown in Plates 1 and 2. For storage the heads are dried by long extraction with several changes of alcohol, at room temperature, before finally suspending in ether and filtering with suction.

The above method is applicable to salmon and herring sperm without modification. Some



PLATE 1

Salmon Sperm Heads  $\times 700$

(Stained methylene blue.)

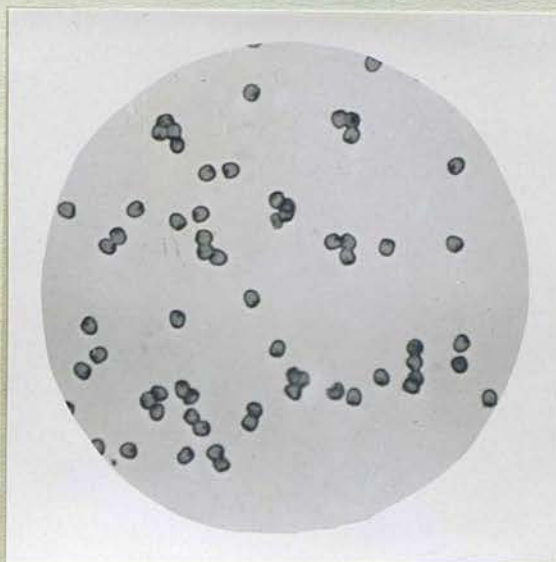
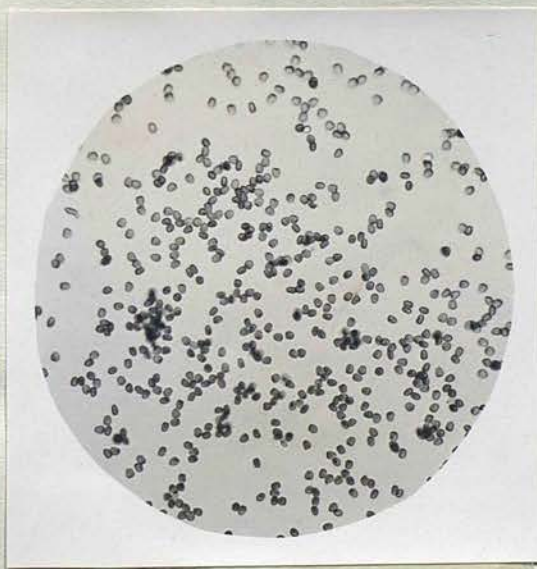


PLATE 2

Salmon Sperm Heads  $\times 300$

(Stained methylene blue.)



difficulty is, however, experienced in the preparation of cod sperm heads. This is due to the more complex structure of cod testes as compared with those of salmon and herring, resulting in the admixture of the sperm heads with extraneous material. A contributory factor is the smaller size of the cod sperm heads which makes the separation of impurities by fractional centrifugation difficult. In order to overcome these difficulties it is necessary to suspend the material obtained from the straining process in 4% acetic acid and to stir the mixture vigorously. This treatment is repeated on the solid material obtained by centrifuging, using 1% in place of 4% acetic acid, until the supernatant fluid is clear and the sperm heads appear to be homogeneous on microscopic examination. As judged by the effect on herring sperm heads (see Stedman and Stedman, 1947) such treatment does not alter their composition significantly.

#### ERYTHROCYTE NUCLEI.

Erythrocytes are peculiar among cells in that they occur in the free state suspended in an aqueous medium, the plasma, which contains relatively few other formed elements. When they are nucleated, as in birds and reptiles, they therefore constitute a readily available source of cell nuclei. Their peculiar mode of occurrence combined with a high concentration of haemoglobin in their cytoplasm, necessitates the use of a special technique for the isolation and purification of their nuclei.



The first more or less successful attempt to isolate nuclei from avian erythrocytes was probably that of Ackermann (1904). The cells were laked by short contact with water to which a solution of NaCl was added in amount sufficient to bring the salt content of the suspension fluid to that of physiological saline. The nuclei were then centrifuged down and washed repeatedly with saline in order to remove haemoglobin. The whole process was repeated a number of times over the course of two days. There is no doubt that Ackermann's product consisted essentially of nuclear material but it is doubtful from his description of it whether individual nuclei were present. A repetition of this procedure has, indeed, shown that contact with water for a sufficiently long period to bring about complete haemolysis of the erythrocytes causes the nuclei to coalesce together until they form a viscous stringy mass in which scarcely a single nucleus can be discerned under the microscope.

Two other methods for laking erythrocytes, namely repeated freezing and thawing, and the use of haemolytic agents such as saponine, have come into common use. When applied to the isolation of nuclei, however, they suffer from the disadvantage inherent in all the methods, that is they fail to bring about complete haemolysis without prolonged action. There always appears to exist in preparations of erythrocytes a fraction which is much more

resistant than the bulk to the haemolytic agent.

In the present investigations nuclei were prepared from fowl erythrocytes by the following method. Blood is collected by bleeding from the carotids and is allowed to flow onto a suspension of sodium oxalate (100mg. per bird) in saline with continuous stirring. It is strained through a layer of muslin to remove any small clots that may have formed, the corpuscles separated from the rest of the plasma by centrifuging and further purified by washing thrice with about three times the volume of saline. Laking is brought about by stirring the corpuscles with an equal volume of saline <sup>containing</sup> ~~made~~ 0.3% of the haemolytic agent (saponine or 'cetavlon'). After five minutes the nuclei and unlysed cells are centrifuged down and washed with saline until relatively free from haemoglobin. At this stage a layer of intact cells always collects at the bottom of the centrifuge tube. The product is therefore again mixed with an equal volume of the lysing agent and left overnight. This has been consistently found to give complete haemolysis while shorter periods of several hours duration have proved insufficient. The nuclei can now be freed completely from haemoglobin by repeated washing with saline after which a suspension of them in saline is strained through a layer of muslin to remove a few small masses of viscous and structureless material. The nuclei, after removal from the saline, are washed first with 50%

alcohol, afterwards with alcohol of increasing concentration and finally with absolute alcohol. The latter usually removes an intense yellow pigment, presumably a carotenoid, and washing is continued until this has been completely extracted. The nuclei are then washed with ether and filtered by suction.

As an alternative to washing with 50% alcohol to remove salt, the nuclei have in some preparations been washed with 1% acetic acid. This not only removes salt but also exerts a slight fixative action on the nucleus.

When examined microscopically the nuclei prepared by the above method appear to be on the whole quite pure. There is, however, always a proportion of nuclei present to which minute fragments of non-staining material can be seen adhering. This presumably represents part of the stroma of the cells which has not been completely removed during the preparation. It is probable that, although there is no evidence for this, the viscous and structureless material removed by straining contains much of the stroma.

#### GLANDULAR NUCLEI.

The method used for the isolation of nuclei from glandular and similar tissue is one which has been used by Stedman and Stedman (see eg. 1947) since 1943. It was evolved from that of Stoneburg (1939) which, in its turn, was based on the observation by Crossman (1937) that when tissue cells are treated



with 5% citric acid the nuclei are <sup>extruded</sup> ~~extracted~~ from them. Stoneburg finely minced tissue, such as heart muscle or tumour, and treated the pulp with 5% citric acid. After a certain amount of purification by mechanical means he removed the remainder of non-nuclear material with pepsin, a procedure previously used by Miescher (1871a) for the isolation of nuclei from pus cells, and one which is almost certain to produce considerable changes in the composition of the nuclei. Stoneburg stated that his method was inapplicable to thymus glands and to liver.

The adaptation used in this investigation, which avoids the use of pepsin and moderates the action of the acid, is as follows.

The tissue (thymus gland, liver, spleen, etc.), obtained from the slaughter house immediately after the death of the animal, is freed from as much connective tissue as possible and minced coarsely in a household mincer. It is then converted into a fine pulp by passage through a 'Latapie' mincer. The pulp is suspended in about four volumes 4% acetic acid and stirred at high speed for about 30 minutes. This stirring is essential to disintegrate the pulp and to bring the cells into intimate contact with the acid, and to be fully effective, must be carried out without delay after mincing the tissue. After standing one or two hours the fluid is strained first through gauze, which removes grosser contaminants, such as coagulated masses of protein, and then through four



layers of muslin. This process removes small pieces of fibre and a variable amount of finely divided slimy material. The latter, if present in appreciable amounts, tends to clog the muslin and renders the process slow. The filtrate from these operations contains the nuclei, usually admixed, particularly in the case of liver, with a few whole cells and a quantity of fine cytoplasmic granules.

Purification of the nuclei is brought about by repeated centrifuging. The nuclei are first separated by centrifuging at 2,000 r.p.m. for 20 minutes when the dense nuclei form a more or less compact layer, followed by a loose layer of cytoplasmic debris and whole cells, with finally a turbid supernatant. These fractions may be separated by the appropriate mechanical means and the nuclei purified by repeated fractional centrifugation in 1% acetic acid until only one layer is present and the supernatant fluid is clear.

Plate 3 shows a preparation of ex liver nuclei at this stage stained with methylene blue.

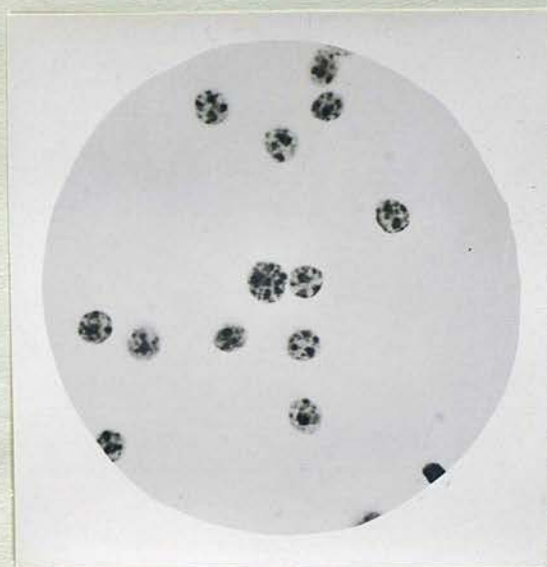
When a satisfactory preparation is obtained, as shown microscopically, the nuclei are washed in alcohol followed by ether as described for sperm heads.

Variations of the above method have been used, such as washing the pulped material in saline, or saline containing 0.3% cetavlon, before the

PLATE 3

Ox Liver Nuclei x700

(Stained methylene blue.)



acetic acid treatment in order to help to remove cytoplasmic proteins, especially ribose nucleoproteins, which are, according to Mirsky (1943), soluble in saline. The results obtained, however, did not justify the great deal of labour and time involved.



## NUCLEIC ACID ESTIMATIONS.

TOTAL NUCLEIC ACID. Total nucleic acid was determined by total phosphorus estimations. This procedure appeared justifiable as no other phosphorus containing compound was known to occur in the nucleus. Recent work by Davidson<sup>(1949a)</sup>, however, suggests that a number of other phosphorus containing compounds may be present, but in the lack of any details it appeared justifiable to assume the phosphorus content to be a measure of the total nucleic acid present. The estimations were carried out by the method of Fiske & Subbarow (1925) using the reagents of King, Haselwood and Delory, (1937).

7mg. nucleic acid dried in vacuo over  $P_2O_5$  for three days at room temperature, were weighed into a 'Pyrex' boiling tube and digested with 0.9ml.  $H_2SO_4$  until all charring had disappeared. The digestion was complete in 18 - 24 hours. The cooled digest was made up to 25ml. and 2ml. aliquots taken for estimation. To the slightly diluted sample is added 1ml. 2.5% ammonium molybdate in 2.5N sulphuric acid, followed by 0.2ml. reducing reagent (containing 1-amino-2-naphthol-4-sulphonic acid). The volume is made up to 10ml. with water and the colour read in the Spekker Absorptiometer using Spectrum Red filters (680m $\mu$ ). Standards were prepared similarly from a phosphate solution using ammonium molybdate in 5N sulphuric acid in order to keep the acid concentration constant.



RIBOSE NUCLEIC ACID. 75mg.nuclei,dried as above,were weighed into a graduated centrifuge tube,suspended in 1ml.water and 1ml.2N KOH added. The suspension was incubated for 18 hours at 37'C,solution taking place within two hours,after which the procedure was exactly as described in the previous section for pure ribose nucleic acid (p.26).

RESULTS.

Table 4 gives the results of the total and ribose nucleic acid estimations for isolated ex nuclei, while Table 5 gives similar values for various other species. Each value is the mean of two or more determinations, differing by not more than  $\pm 5\%$



TABLE 4

52

TISSUE	PREPN.	TOTAL N.A.	R.N.A.
OX LIVER	2/46	29.2%	1.9%
	8/47	28.7	1.3
	2/48	30.5	1.1
	3/48a	30.5	1.0
	3/48b	30.7	0.86
	11/48	28.7	1.5
	12/48	27.5	1.8
	4/49	28.4	1.8
OX THYMUS	10/47	36.8	0.83
	4/48	36.4	1.3
	10/48	34.3	1.0
	3/49a	34.7	0.86
	3/49b	35.4	0.80
	3/49c	35.1	0.84
	3/49d	34.4	0.64
OX SPLEEN	10/46	33.5	0.70
	10/47	33.6	1.1
	12/48	32.6	0.78

Total N.A. = Nucleic acid by phosphorus estimation.

R.N.A. = Ribose nucleic acid determined by  
furfural estimation.

SPECIES	TISSUE	PREPN.	TOTAL N.A.	R.N.A.
RABBIT	LIVER	II/48	26.2%	2.0%
	KIDNEY	II/48	26.0	I.2
FOWL	ERYTHROCYTE	IO/47s	33.9	2.3 *
		8/48s	34.8	2.5 *
		I2/48s	34.2	2.2 *
		2.48c	38.1	0.74
		3/48c	37.8	0.88
	LIVER	3/48	31.2	2.0
		I2.48	29.4	2.2
	THYMUS	IO/48	36.3	I.3
		I2/48	32.0	I.4
COD	SPERM	3/49	30.3	0.3
HERRING	SPERM	2/48a	59.0	0.0
		2/48b	40.1	0.2
		3/48a	45.2	0.2
		3/48b	38.8	0.2
SALMON	SPERM	S.P.	59.5	0.1
		S.K.	60.8	0.1

Legends as in Table 4.

\* These are abnormal values brought about by laking  
with saponine - see page 66.



# CHROMATOGENIC SUBSTANCES OTHER THAN RIBOSE NUCLEIC

ACID. The possibility that pentoses, pentose nucleotides, glucuronides, glycogen and mucoproteins, if present in sufficient concentration in cell nuclei, might contribute to the yield of furfural under the conditions used in the foregoing estimations has been discussed in Section A (p.30). The following tests were therefore carried out.

Using the method of Roe and Rice (1948) free pentoses could not be detected in a 100mg. sample of ox liver nuclei. Similarly glucuronides could not be detected by Tollen's naphthoresorcinol reaction as described by Hanson et al.(1944), while glycogen was shown to be absent from ox liver <sup>nuclei</sup> by the technique of van Wagtenork (1946). Dounce (1943g) also claimed that his preparations were free from glycogen, using rat liver.

The position regarding co-enzymes and mucoproteins is less satisfactory, however, There is at present little evidence to suggest their presence but equally, there is nothing to disprove it. Co-enzymes especially are a probable nuclear constituent, although doubtlessly present, as indicated by Dounce (1943c), in very small amounts.

It is probable, therefore, that the ribose nucleic acid content of isolated cell nuclei as measured by furfural formation is essentially a correct value, but it may be slightly high because of the presence of small amounts of <sup>other</sup> furfural yield-

ing substances, such as co-enzymes and mucoproteins.

### PHOSPHORUS ESTIMATIONS.

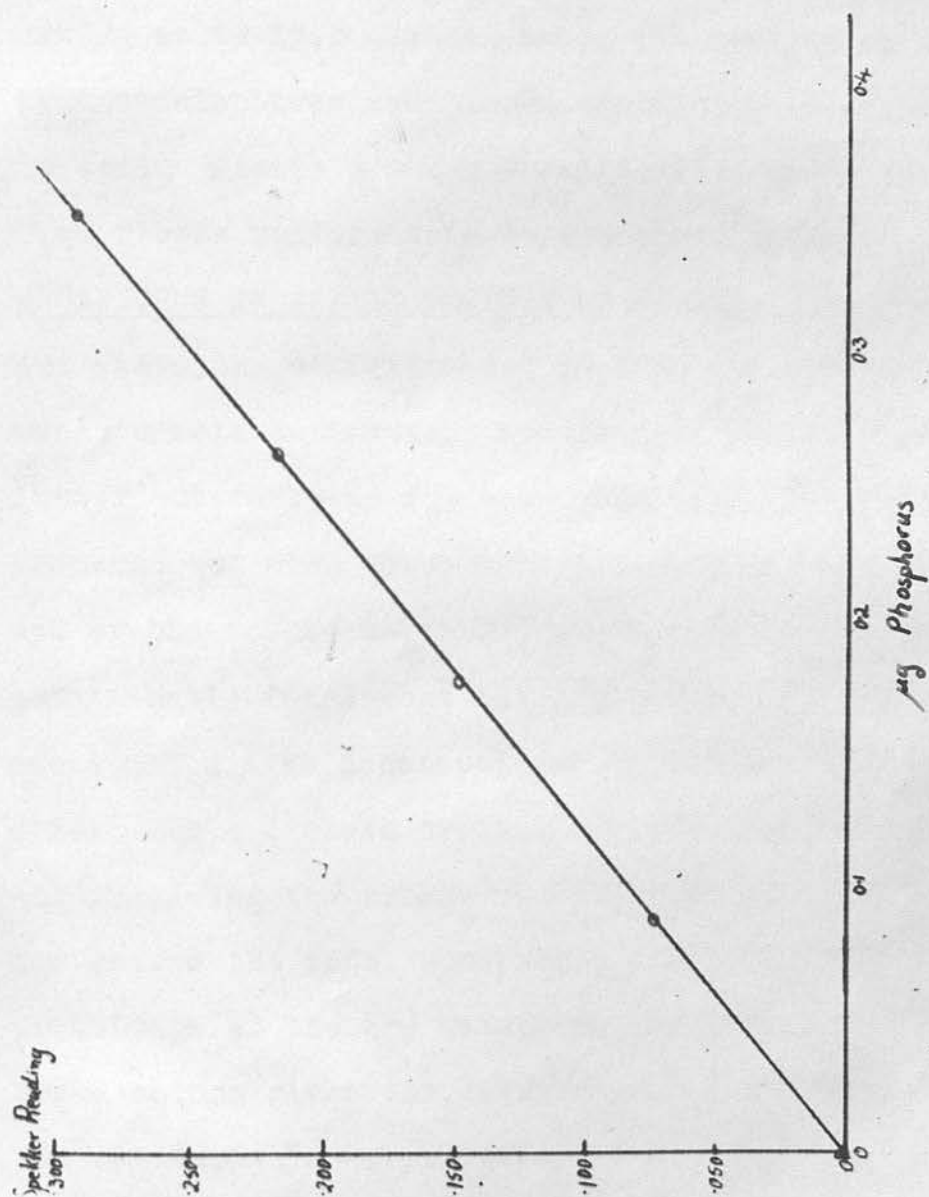
In view of the relatively small amounts of ribose nucleic acid apparently present, it was thought desirable to check the results obtained using the furfural method previously described by means of phosphorus estimations. According to Schmidt and Thannhauser (1945) the centrifugate used in the ribose nucleic acid test should have contained only the latter substance in the form of nucleotides, there being no evidence to suggest the presence of phosphoproteins in the nucleus. Davidson et al. (1949) have, however, since produced evidence that such compounds do occur.

When the phosphorus content of the centrifugate was determined by the method described below in all cases there was found to be present more phosphorus than could be accounted for by the ribose nucleic acid, assuming, as appeared justifiable, the results of the furfural estimation to be correct.

PHOSPHORUS ESTIMATION. The method of phosphorus estimation previously described (p.49) suffered from the disadvantage of being slow. It was therefore modified by the use of perchloric acid as oxidising agent for the removal of organic matter (see King, 1946). A 1ml. aliquot of the solution under investigation was digested with 1.2ml. 60% perchloric acid in a Folin-Wu sugar tube until all charring had ceased, the process usually taking about 15 minutes. The cooled digest was diluted to about 10ml. with

- RIBOSE NUCLEIC ACID PHOSPHORUS -

Fig 8





water, when a white crystalline precipitate of potassium perchlorate appeared. As this did not adsorb any of the colour developed later it was ignored and colour development brought about by the addition of 1ml. 2.5% ammonium molybdate in water, followed by 0.5 ml. naphthalenesulphonic acid reducing agent before making up to 12.5 ml. with water and reading as before against standards and blanks containing 1ml. perchloric acid. Figure 8 shows a calibration curve prepared from ribose nucleic acid by the above method.

PHOSPHORUS IN ALKALI DIGESTS OF NUCLEI. As pointed out above, the centrifugates used in the ribose nucleic acid estimations should have contained the latter substance as the sole phosphorus containing compound but when phosphorus estimations were carried out by the method described above, it was ~~considerably~~ consistently found that the phosphorus detected could not all be accounted for by ribose nucleic acid. Table 6 shows typical results, the first column giving the ribose nucleic acid phosphorus and the second the total phosphorus, both expressed as a percentage of the dry weight of the nucleus. The third column gives the difference between the above two values, again as percentage dry weight of nucleus.

Table 6.

Nuclei.	R.N.A.P. %	Total P. %	Diff. %
Ox liver, 11/48.	0.15	0.25	0.10
12/48.	0.16	0.28	0.12
Ox thymus, 10/47.	0.08	0.18	0.10
4/48.	0.11	0.20	0.09
Ox spleen, 10/46.	0.06	0.17	0.11
12/48.	0.07	0.16	0.09
Fowl erythrocytes, 2/48.	0.06	0.13	0.09
3/48.	0.08	0.15	0.07
Herring sperm, 4/48a.	0.01	0.13	0.12
4/48b.	0.01	0.11	0.10

These results show quite conclusively that there was more phosphorus present in the centrifugate than could be accounted for by ribose nucleic acid, the extra phosphorus varying in amount, but representing from 0.07%- 0.12% dry weight of nucleus, or calculated as nucleic acid, roughly 0.8%- 1.2% of the nucleus.

Deoxyribose nucleic acid was known to be absent from the centrifugates by their negative Dische reactions, as mentioned previously. The 'extra' phosphorus must therefore have been due either to decomposition products of deoxyribose nucleic acid which did not give the Dische reaction, or to some non-nucleic acid phosphorus compound present in the nucleus.

The matter was further investigated in ox thymus nuclei. Samples of nuclei were treated with alkali as in the estimation of ribose nucleic acid and were then precipitated with 0.4ml. 6N HCl

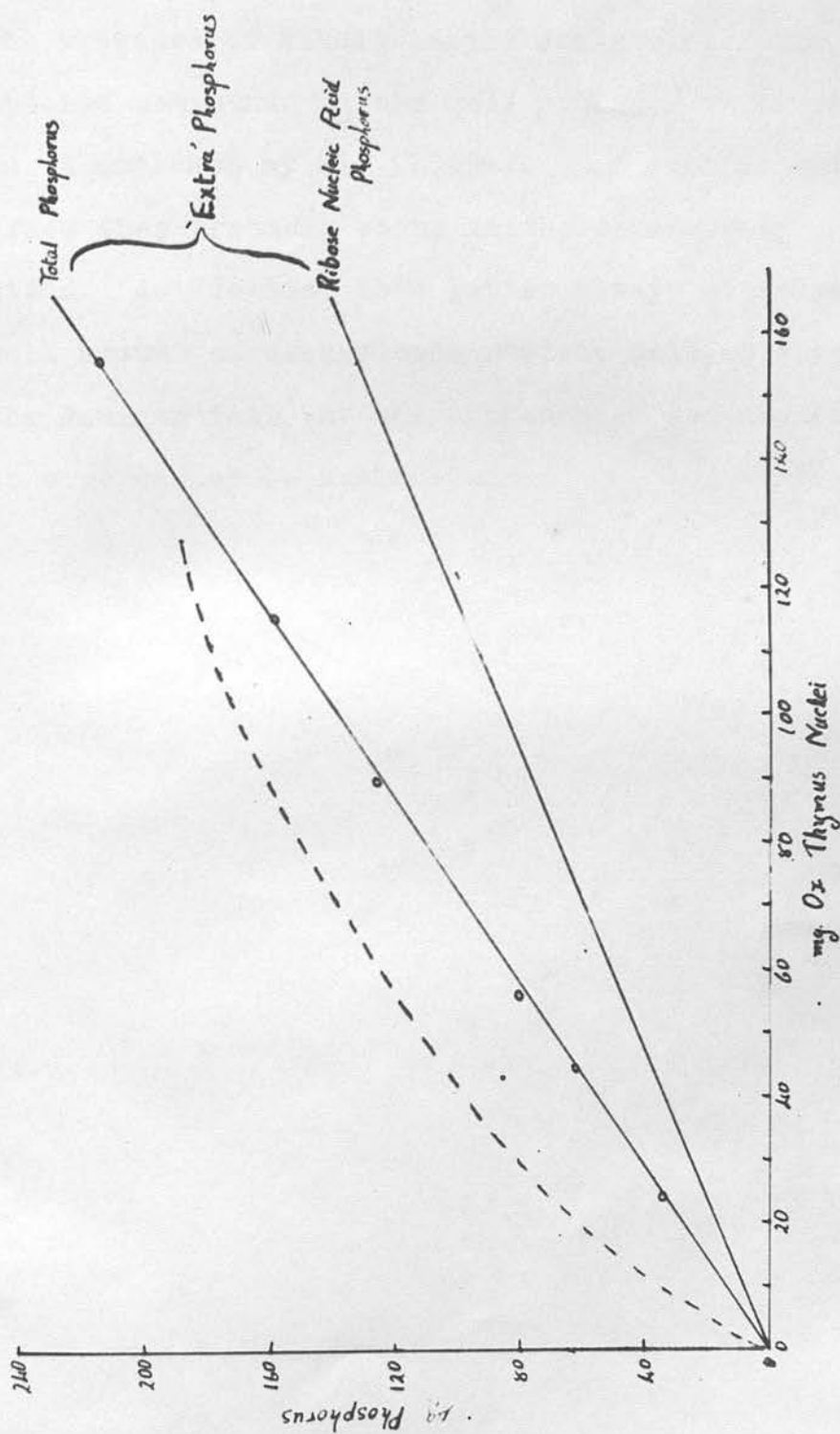
followed by 2ml. 0.1N HCl, this modification of technique being adopted to keep the volume of fluid constant over a wide range of weights of acid nuclei. Figure 9 shows the liberation of acid soluble phosphorus from such nuclei, and also the ribose nucleic acid phosphorus, calculated from the furfural results.

Attempts were made to investigate the possible destruction of deoxyribose nucleic acid by alkali treatment. The specimens of nucleic acid were ~~from~~ <sup>from</sup> a Neumann preparation ~~of~~ <sup>a specimen</sup> herring sperm, and ~~from~~ <sup>from</sup> cod sperm prepared by extraction with 0.2% NaOH in 10% sodium acetate after removal of histone. The amount of phosphorus liberated when the above nucleic acids were treated with alkali was very much greater than that liberated from the corresponding amount of nucleic acid in cell nuclei or nucleohistone, as is indicated by the dotted curve in Fig. 9. The addition of histone to the nucleic acid made no significant difference to the liberation of acid soluble phosphorus from the latter on alkali treatment, suggesting that the striking difference between free deoxyribose nucleic acid and the same substance in cell nuclei was due to some change in its nature brought about by the preparation.

As this problem was incidental to the main project further investigations were not carried out but the results obtained showed conclusively that the Schmidt and Thanhauser technique could not be

- 'EXTRA' PHOSPHORUS FROM THYMUS NUCLEI -

Fig. 9





to material such as cell nuclei without considerable modification when the estimations were based on phosphorus determinations. The results also suggested the presence of alkali-labile non-nucleic acid phosphorus compounds in the cell nucleus, in confirmation of Davidson et al. (1949a). If such compounds do exist they probably occur in the chromosomal fraction. As isolated this latter always contains a small amount of deoxyribose nucleic acid, as shown by the Feulgen test, but the presence of non-nucleic<sup>acid</sup> phosphorus cannot be excluded.

## DISCUSSION.

The discussion of the location of the ribose nucleic acid and its physiological significance will be left until Section D. In the present section only the meaning of the small, but significant, amounts of ribose nucleic acid present in the cell nuclei isolated by the available procedures will be considered.

### NATURE OF ISOLATED NUCLEI.

The first question of interest is the relationship of isolated cell nuclei to the nuclei of the living cell. In this investigation, the main method used for the isolation of nuclei is of a fairly mild nature, the most drastic treatment being contact for a few hours with 4% acetic acid (0.66N; pH2.4) followed by more or less prolonged contact with 1% acetic acid (0.17N; pH2.7). This treatment undoubtedly alters the finer structure of the living nucleus in a way entirely analogous to that brought about by the more gentle cytological fixation techniques. For the present investigation this is of little importance as it is extremely unlikely that the nature or quantity of the nucleic acids is altered in any way by such treatment. That no drastic change to the protein constituents can have occurred is shown by isolated nuclei exhibiting a fair degree of  $\beta$ -glucuronidase activity when tested by the micro-method of Kerr et al. (1949), so that complete denaturation cannot have taken place.

Pollister et al. (1949) claimed that the isolation of cell nuclei caused great changes in their composition, mainly brought about by the removal of protein. As this claim was based on Pollister's histochemical methods (see Pollister and Ris, 1947) it is of only doubtful value owing to the many criticisms which can be levelled at so called quantitative histochemistry. Thus, he used the Feulgen technique as a quantitative measure of the deoxyribose nucleic acid present in the nucleus : as some 30% of the phosphorus is removed during the estimation (Stedman and Stedman, 1949) it is obvious that the technique can in no sense be considered quantitative. The method of protein estimation is again of doubtful value because of the complexity and vigour of the conditions of the method, which involves the use of hot trichloroacetic and of Millon's reagent. In view of these criticisms it seems justifiable to consider the claims of Pollister with reserve. Again Pollister worked with physiological saline : he claimed that such treatment removed non-histone protein from frozen sections. This work is therefore not directly applicable to the present investigation where the nuclei are fixed by the preliminary 4% acetic acid treatment. Dounce (1943a,b) also suggested that isolation of nuclei at a pH approaching neutrality caused removal of protein but pointed out that this did not occur with nuclei isolated in acid solution, as in his citric acid method.

Plate 3 shows a typical preparation of ox liver nuclei. These show an appearance similar to a normal cytological liver preparation, that is, a typically spherical nucleus with a fairly dense chromatin network and one or more prominent nucleoli. This fact alone is good evidence that no great change has occurred during the isolation procedure. In this connection it is probably not without interest that the majority of cytological fixatives used in the study of cell nuclei, on which study much of the theory of cytogenetics is based, contain large amounts of acetic acid; eg. Carnoy, with 25% acetic acid; Bouin and Zenker, both with 5% acetic acid, are commonly recognised fixatives giving 'good' nuclear preparations. Again Caspersen and Schultz (1940) in their study of the nucleolus, macerated root tip cells of SPINACIA in 45% acetic acid for several days. The effect of such treatment must be very detrimental to the cell considering that a three hour treatment with acetic-alcohol (25% acetic acid) removes 10% of the histone from isolated cell nuclei (Stedman and Stedman, 1948).

As already mentioned, a possible criticism of the results is that substances have been removed from the nuclei during isolation. There is certainly little doubt that any substance of low molecular weight present in the living cell nucleus would be at least partly removed. This may or may not include co-enzymes depending on the strength of



the link between the appropriate co- and apo-enzyme. That any substance of high molecular weight is lost is unlikely, however. As pointed out, Dounce (1943 a, b) suggested that at pH6 non-histone protein was removed from the nucleus, at pH2 or less he believed that histone was extracted, while at slightly acid reaction (pH3) the composition of the nucleus was apparently unchanged during the isolation. There is little to suggest that histone is removed during isolation in the present investigation, as, apart from the ~~consistency~~-constancy of the composition of a large number of different specimens of nuclei from various sources, even prolonged treatment of isolated nuclei with 4% acetic acid removes only minute amounts of material precipitable by the addition of sulphuric acid and a large excess of alcohol.

One of the purposes of the present work was to study the nature of the nucleolus : it is of importance, therefore, that the staining properties of that organelle are not altered during isolation. According to Brachet (1942) the nucleolus of liver cells stains red when treated with a mixture of methyl green and pyronine. Isolated ox liver nuclei were smeared from 1% acetic acid, washed, stained overnight in methyl green-pyronine, washed and differentiated 30 seconds in alcohol before mounting in balsam. In such preparations the nucleolus stained a deep red-purple and the nuclear sap a pale blue. An identical preparation was obtained when fresh

mouse liver was smeared, fixed in Carnoy, and stained as above. It is therefore obvious that the pyronine staining material of the nucleolus, be it ribose nucleic acid or not, is certainly not removed during the isolation of the nuclei.

#### UPTAKE OF MATERIAL DURING ISOLATION.

The possibility that during the isolation of the nuclei material is taken up from the media must next be considered.

In the case of fowl erythrocytes there is definite evidence that such a process can take place. As is obvious from Table 5, the ribose nucleic acid <sup>contents</sup> of isolated fowl erythrocytes <sup>nuclei</sup> appears to fall into two groups, one group having a ribose nucleic acid content of about 2.5% (preps. 10/47, 8/48 and 12/48) and the other a content of about 1% (preps. 2/48, and 3/48). In the first group the laking agent used was saponine and in the second, cetavlon. The much higher apparent ribose nucleic acid content of the saponine laked specimens suggested that contamination of the nuclei with the latter substance had occurred. The two samples of saponine used both gave high furfural yields when submitted to the usual treatment. It seems certain, therefore, that contamination of the nuclei had occurred, and in such a way that even prolonged washing could not entirely remove the contaminant.

It is thus obvious that contaminants yielding furfural form a potential source of error

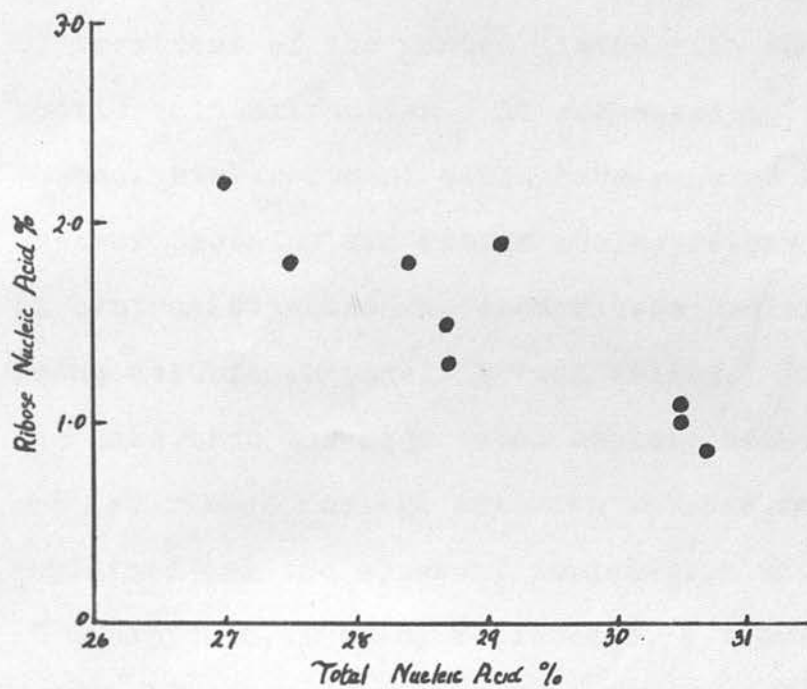
in the determination of ribose nucleic acid in isolated nuclei, and also obvious that great care is required in assessing the significance of any substance supposedly present in small amounts in isolated nuclei.

Closely related to the above is the question of whether the values for the ribose nucleic acid content of isolated cell nuclei represent true values for the nucleus or whether they are apparent values brought about by varying degrees of cytoplasmic contamination. Gross contamination of the nuclei is ruled out by their microscopic appearance, but contamination by adsorption is a possibility which must be considered. Especially is this the case in the liver where contamination is very likely because of the large amount of ribose nucleic acid present in the cytoplasm. In the other tissues examined contamination in this way is not so probable.

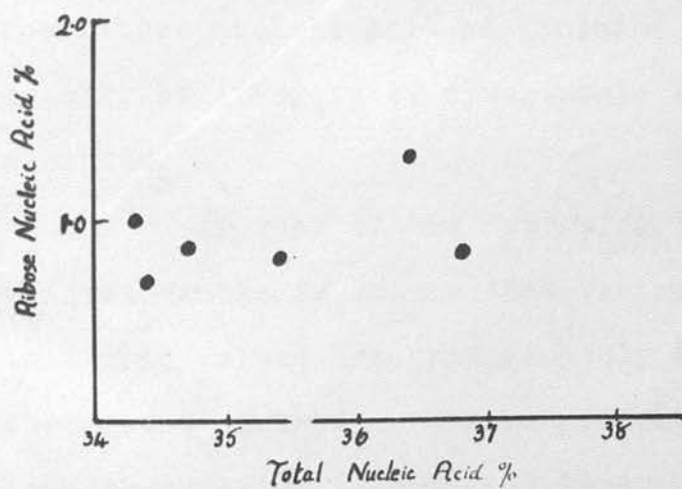
Figure 10 shows a correlation diagram for the total and ribose nucleic acid contents of several specimens of isolated ox liver nuclei. There is apparently an association of a high ribose nucleic acid content with a low total nucleic acid. One explanation is that part of the apparently nuclear ribose nucleic acid is actually cytoplasmic contamination, which would have the effect of increasing the ribose nucleic acid content of the preparation while lowering the total nucleic acid. because of the lower total concentration of the

- NUCLEIC ACIDS IN OX NUCLEI -

LIVER - Fig. 10



Thymus - Fig. 11





latter in the cytoplasm. In Fig.10,however,the three specimens of nuclei with total nucleic acid contents of roughly 30.5% and ribose nucleic acid contents of 1% may be abnormal. These specimens were prepared by treatment of the minced tissue with saline before acetic acid extraction. As suggested by the work of Dounce,this treatment could have removed some of the nuclear proteins and ribose nucleoprotein,the latter, in contradistinction to ~~deoxy~~ribose nucleoproteins, being soluble in physiological saline. If this be the case,then the high total nucleic acid content and low ribose nucleic acid content are readily explained and the apparent correlation shown in Fig. 10 disappears,it being replaced by a figure explicable as the expected variation from preparation to preparation,as in the case of thymus nuclei (Figure11). Be this as it may,however,the possibility that some of the ribose nucleic acid of isolated nuclei,and especially of liver,is of cytoplasmic origin cannot be excluded.

In view of the foregoing discussion it seems justifiable to assume that the reported ribose nucleic acid values are approximately true estimates of the amount of that substance present in the living nucleus,there being no evidence to suggest the loss of ribose nucleic acid,or indeed,any substance of high molecular weight,during the isolation of the nuclei. Similarly there is no evidence of contamination,except in the case of saponine laked fowl erythrocytes,although traces of adsorbed cytoplasmic

ribose nucleic acid cannot be excluded. It must be noted, however, that the values for the ribose nucleic acid content reported must be the maximal values brought about by the presence of other furfural yielding substances than ribose nucleic acid and from contaminating substances in the cytoplasm. In the case of fish sperm heads especially, the small apparent ribose nucleic acid content may well be due to this effect.

#### SIGNIFICANCE OF 'EXTRA' PHOSPHORUS.

The 'extra' phosphorus discussed on p. 58 would appear to be either a decomposition product of deoxyribose nucleic acid or of some other phosphorus compound present in the nucleus. This phosphorus is, however, present in amounts small enough to justify the assumption made in this work that the total phosphorus of the nucleus is a measure of its nucleic acid content. As pointed out, the 'extra' phosphorus, expressed as nucleic acid, amounts to less than 1% of the nucleus which is of little significance with regard to total nucleic acid contents of the order of 30% as reported in this investigation. The procedure adopted for the determination of total nucleic acid in the previous section is therefore completely justifiable.

Schmidt and Thannhauser (1945) claimed that their method, on which the present work is based, gave an accurate measure of the nucleic acid content of a tissue, basing their claim on the quantitative

recovery of added ribose nucleic acid from acetone dried tissue powders. They also stated that their determinations of ribose nucleic acid phosphorus agreed with estimations carried out by Majbaum's (1939) method. This would suggest that the effect noted in the present work, that is, the appearance of 'extra' phosphorus, only arises in the case of cell nuclei in which the material is presumably located, and in which the concentration of ribose nucleic acid is very low. As already indicated this problem was incidental to the main investigation and was therefore not considered further. The evidence available, however, would suggest that this 'extra' phosphorus was probably identical with the phosphoprotein phosphorus shown by Davidson et al. (1949a) to be present in isolated cell nuclei and also in minute amounts in whole tissues, (Davidson et al. 1949b) where the source is no doubt the nuclei contained therein.

SECTION C.

Nuclear Ribose Nucleic Acid in Proliferating  
Tissue.



## INTRODUCTION.

It has for some time been recognised that the ribose nucleic acid content of an organ is connected with its state of activity. Schneider (1947) showed an increased ribose nucleic acid content of rat and mouse tumour as compared with the normal tissue. Novikoff and Potter (1948) showed a rise in the ribose nucleic acid content of regenerating rat liver, while Caspersen and his associates, in a long series of papers (summarised by Caspersen in 1947) have shown that the cytoplasm of actively dividing cells or of cells engaged in protein synthesis is rich in ribose nucleoproteins. It thus seems fairly definite that the ribose nucleic acid content of a proliferating tissue is higher than in a resting tissue. This work, however, has been carried out almost entirely on whole tissues or on cytological preparations, and little work has been reported on the nuclei isolated from such tissues.

Darlington (1942) stated that nucleoli were large in protein producing cells, such as egg, meristematic, or tumour cells, and implied that a large amount of ribose nucleic acid was present in such nucleoli. Koller (1943) claimed that tumour cells contained a large number of Feulgen negative nucleoli and that the nucleic acid content of such cells attained abnormally high values: these claims were based on cytological study of tumours using the Feulgen technique as a means of detecting deoxyribose

nucleic acid. From such work Koller concluded that a rise in deoxyribose nucleic acid concentration was the fundamental cause of malignancy. Apart from such work, little has been reported on the ribose nucleic acid content of the nuclei of proliferating cells, although Dounce (1943b) gave values for the deoxyribose nucleic acid content of isolated rat hepatoma nuclei, which had a lower figure than normal, contradicting the claims of Koller.

Liver was found to be the most suitable organ in which to carry out an investigation of the nucleic acid content of nuclei isolated from proliferating tissue as it <sup>is</sup> readily available in large amounts from the normal laboratory animals and cell proliferation is easily induced by partial hepatectomy or by administration of liver poisons such as carbon tetrachloride.

This section is therefore concerned with the ribose nucleic acid content of nuclei isolated from regenerating liver tissue. The results obtained with nuclei isolated from other tissues in cases of leukaemia in man are also mentioned, although the latter are difficult to interpret because of the lack of adequate normal controls.

## METHODS.

RAT LIVER. Regeneration was brought about by partial hepatectomy. The animals used were male and female Wistar rats of 200-250gm. weight fed on standard rat cake (high protein content). For 24 hours before and after the operation the animals were allowed only 10% glucose in water. Atropine (13mg./100gm.) in phosphate buffer pH7 was given by subcutaneous injection 30 minutes before anaesthetising with ether. The operation was carried out using the technique of Higgins and Anderson (1931).

The medial and left lateral lobes were exposed through a mid-line ventral incision extending 1.5cm. caudally from the xiphoid process and removed after double ligature with silk. No bleeding was caused, the only loss of blood being that left in the excised lobes of liver. After introducing 100-200mg. sulphanilamide in warm saline into the abdominal cavity the muscle layer and skin were closed independently with discontinuous nylon sutures. The wound was disinfected daily with 1/1000 acriflavine.

The mean mortality was less than 20% and was due almost entirely to respiratory troubles aggravated by the anaesthetic - in one batch the mortality was 70% due entirely to pathological changes in the lungs. It appeared obligatory to use ether as anaesthetic in spite of its effect on the respiratory tract as it was impossible to use

'Nembutal' and similar liver-detoxicated barbiturates when 60%-70% of the liver was removed during the operation.

MOUSE LIVER. The liver was poisoned by injection of 50% carbon tetrachloride subcutaneously as a solution in olive oil, 0.2ml./30<sup>gm</sup>. Regeneration had commenced within 48 hours.



## RESULTS.

Nuclei were isolated from the normal and proliferating tissues by the methods previously described . No great difficulty was met with even in the early stages of carbon tetrachloride poisoning when considerable amounts of fat were present. As the amount of material was small, the time of preparation was much reduced, a preparation of rat liver nuclei being finished within 6 hours of the death of the animals.

HISTOLOGY. In the mouse, according to Levvy, Kerr and Campbell (1948), regeneration after  $\text{CCl}_4$  poisoning is well advanced after 3 days and is almost complete after 7 days.

In the rat a similar picture was obtained from haematoxylin-eosin stained preparations during the course of this work. At 3 days the number of nuclei per field was less than normal but mitoses were visible; at 4 days much mitotic activity was apparent and the number of nuclei greater. By 8 days the liver was apparently almost back to normal. At all stages the Kupfer cells were very prominent. This would suggest that the most active cell division was occurring at 3-4 days after partial hepatectomy and that the apparent 'growth' of the residual liver prior to this was due to the uptake of water or some form of increase in weight without cell division.

ANALYTICAL RESULTS. The analyses were carried out by the methods described in the previous sections, and are reported in Table 7 for the rat and Table 8

for the mouse. The rat results are from groups of approximately eight animals and the mouse results for groups of twenty.

Table 7.

Prepn.	Normal		Days.	Regenerating	
	Total N.A.	R.N.A.		Total N.A.	R.N.A.
RL.1.	25.4%	4.5%	-	-	-
RL.2.	27.3	2.9	6	27.5%	4.0%
RL.3.	26.5	3.6	4	26.3	4.4
RL.4.	26.4	4.0	3	26.1	4.9
RL.5.	27.5	3.8	3	24.2	5.6
RL.6.	27.3	3.6	3	26.5	5.3

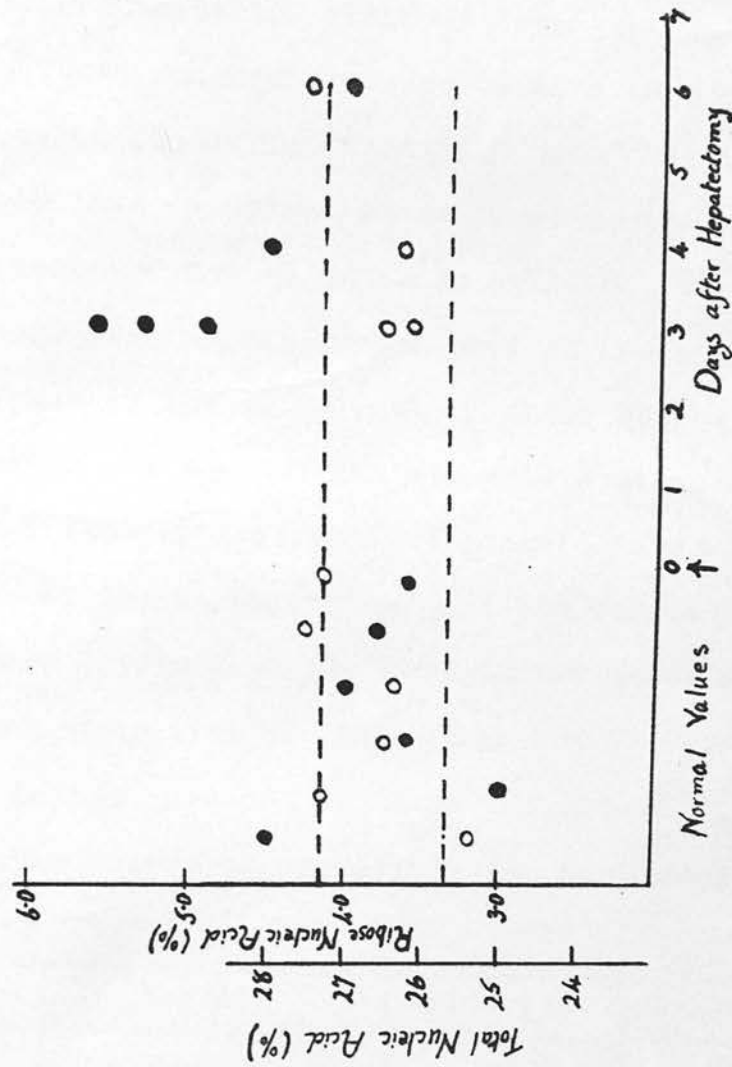
Table 8.

Prepn.	Normal.		Days	Regenerating.	
	Total N.A.	R.N.A.		Total N.A.	R.N.A.
ML.1.	27.9%	3.3%	3	27.5%	3.5%
ML.2.	26.0	3.9	6	27.5	3.2
ML.3.	27.0	3.2	9	27.8	3.0

The most striking feature of the above results is the high figure for the ribose nucleic acid content of normal rat liver,  $3.75 \pm 0.2\%$ , compared with the values for ox, rabbit and fowl nuclei previously reported. The ribose nucleic acid content is also elevated in the regenerating tissues, as shown in Figure 12, which shows the relationship of the ribose nucleic acid content to the state of proliferation of the organ. The dotted lines indicate the mean normal ribose nucleic acid content  $\pm$  twice the standard error of the mean. This value has no statistical significance but it indicates the spread of the normal results.

# REGENERATING RAT LIVER NUCLEI -

Fig. 12



● Ribonucleic Acid (%)  
○ Total Nucleic Acid (%)

Dotted lines show mean Ribose Nucleic Acid content  $\pm 2 \times$  standard deviation of mean

As is seen from Table 8 the ribose nucleic acid content of normal mouse nuclei is again high - approximately 3.5%.

Table 9 gives the results for the few human tissues studied. The nuclei were isolated from post-mortem material obtained from the Holt Radium Institute, Manchester. The tissues were preserved in solid CO<sub>2</sub> during transit. Such freezing was shown to have no effect on ox liver controls - a specimen preserved for 48 hours in solid CO<sub>2</sub> showed no difference from normal in the ease of isolation and appearance of the nuclei, nor in their composition with regard to histone and nucleic acid content.

The preparation of the nuclei from these tumours was by the normal method and was carried out without great difficulty. In some tumour material, however, the preparation of nuclei was almost impossible owing to the presence of large amounts of fibrous material which prevented the separation of the nuclei.

Table 9.

Tumour.	Tissue.	Total N.A.	R.N.A.
Leukaemia.	Spleen.	%	1.2%
Follicular reticulosis.	Liver.	27.8	2.0
	Spleen.	30.5	0.83
	Lymph glands.	34.7	1.0

Unfortunately normal tissues were not available as controls but the results previously published by Stedman and Stedman (1947a) suggests that the total nucleic acid content of tumour cells



does not vary greatly from that of normal tissues. Human liver nuclei contain approximately 27.5% nucleic acid and thymocyte nuclei approximately 36.5%. This figure is probably very near to that for lymphocytes, while spleen nuclei in general have a lower nucleic acid content than the corresponding thymocytes.

### DISCUSSION.

The high ribose nucleic acid content of normal rat and mouse liver nuclei is of interest and is a further example of the ever present danger of generalising from one species to another distantly related species. This rather high content of ribose nucleic acid - 3.8% as against 2% in the ox - may well be connected with the different microscopic appearance of the two types of nuclei. Ox nuclei are shown in Plate 1; rat nuclei, on the other hand, show a granular appearance not present in the ox and in general the nucleolar system is more highly developed in the rat. Rat liver nuclei are much more heterogeneous than ox preparations. This difference in appearance is not so striking in normal histological preparations.

The results quoted in Table 7 and Fig. 12 show a definite increase in the ribose nucleic acid content of regenerating rat liver nuclei as compared with normal nuclei. The maximum value would appear to be reached at about 3 days after the operation, at which time the regenerative processes are commencing, and the value would seem to be nearly normal again at 6 days, when the rate of regeneration has slowed considerably. This distribution is similar to that obtained by Novikoff and Potter (1948) in their study of nucleic acid in whole rat liver during regeneration. These workers found a sharp

rise in the ribose nucleic acid content at 3 days after partial hepatectomy. Davidson and Waymouth (1944c), on the other hand, found no rise in liver nucleic acid under these conditions but apparently based their claim on a small number of experiments.

It is interesting to note that there is no significant change in the total nucleic acid content of liver nuclei during regeneration, that is, the deoxyribose nucleic acid content must drop as the ribose nucleic acid content rises. This could be taken as indicating the transformation of deoxyribose nucleic acid into ribose nucleic acid, that is, the reverse transformation to occur to that postulated by Brachet during ECHINUS development (see Brachet, 1945) and by Mitchell (1942) in the normal **course** of nucleic acid metabolism. There is, however, no evidence to suggest that the ribose-deoxyribose nucleic acid change can take place in either direction and it would therefore be valueless to theorise further on scanty results available. It should be noted that the nucleic acid contents are quoted as percentages and not as absolute amounts present per nucleus. If it were possible to express the results in the latter manner the suggestive parallelism between deoxyribose and ribose nucleic acid might well be shown to be erroneous. In a few cases attempts were made to determine the absolute amounts of nucleic acid by counting the isolated nuclei. The technique was not satisfactory as the high density of the nuclei made

it impossible to obtain an even suspension while the actual size of the nuclei varied so much that it appeared that no great reliance could be placed on the determination of nuclear weights by such methods.

The results obtained with rat liver nuclei may thus be summarised; normal rat liver nuclei contain about 3.7% ribose nucleic acid which in the early stages of regeneration increases to about 5% while the total nucleic acid remains approximately constant.

It should be noted that these results were obtained from nuclei isolated from whole livers, that is, from all types of cell ranging from actively dividing cells to normal resting cells. It may well be, therefore, that the changes described would be greatly accentuated if it were possible to isolate the nuclei from only the actively dividing cells.

With regard to the mouse liver results, nothing can be said. The number of determinations are too few to allow any conclusions to be drawn. In only one of the reported estimations, namely, the ribose nucleic acid content of nuclei isolated from liver 3 days after administration of  $\text{CCl}_4$ , could any departure from normal be expected, at least on the basis of the rat results. No such change was noted. This matter could not be investigated further without the use of large numbers of mice which, at the time did not appear justifiable.



Similarly, the results of the tumour *nuclei* show little of interest, their nucleic acid content being apparently normal. This is contrary to the claims of Caspersen et al. (1942) and of Koller (1943) both of whom claim an increase in the nucleic acid content of tumour cells. They agree, on the other hand, with the work of Dounce (1943b) who detected little difference between normal rat liver <sup>nuclei</sup> deoxyribose nucleic acid and that of Walker tumour *nuclei*. Hepatoma 31 *nuclei* had, however, a lower content of deoxyribose nucleic acid than normal. It is of interest that Dounce, using his citric acid method of isolation, (1943a, b) obtained normal rat liver *nuclei* with a deoxyribose nucleic acid content of 20%-24% (Dische reaction). This compares favourably with the preparations obtained in the present investigation which again had a deoxyribose nucleic acid content of 20%-24%, taken as the difference between the total and ribose nucleic acid content reported in Table 7.

It would not be wise to lay too much stress on the reported tumour results, however, as they apply to types of leukaemia in which the lymphocytes, as isolated, may well be normal, except in that they are present in very large numbers, the actual tumour cell being perhaps one of the lymphocyte precursors, in which case no peculiarities could be expected in the nucleic acid make up of the cells examined.

SECTION D.

General Discussion and Histological Aspects.

The results reported in the previous sections may well be summarised at this point.

Table 10.

Tissue.	R.N.A. content.(%)	R.N.A. as % total N.A.
Ox liver.	1 - 2	3.5 - 6.0
spleen.	1	3
thymus.	1	3
Rabbit liver.	2	7.5
kidney.	1.5	6
Rat liver.	3.8	14
Mouse liver.	3.5	13
Fowl erythrocyte.	0.8	3
liver.	2.0	8.5
thymus.	1.5	4.5
Herring sperm.	0.2	-
Salmon sperm.	0.1	0.5
Cod sperm.	0.3	1.5

It is obvious that these figures although small are by no means negligible and that they are in reasonable agreement with the values published by Vendrely (1948) and by Davidson et al.(1949), which have already been quoted, in Table 1. These latter values would appear to be the only estimates of the ribose nucleic acid content of isolated nuclei on which any reliance can be placed.

As pointed out in the introduction, it is generally assumed that the nucleolus contains ribose-nucleoproteins. It is important, therefore, at this point to review the evidence for this claim before going on to discuss the above results further. The

evidence is conveniently divided into two groups - firstly that based on ultra-violet spectroscopy and secondly, that based on staining reactions. There is, however, considerable overlap in the two techniques.

HISTOCHEMISTRY OF THE NUCLEOLUS. In general, the nucleolus is an acidophilic organelle, staining with eosin in the usual haematoxylin-eosin preparations, and is almost always Feulgen negative. Under certain conditions, however, it will stain with basic dyes, as with pyronine in the Unna-Pappenheim technique, or with safranin in Flemming's Safranin-Gentian violet-Orange G mixture (Wilson, 1928). Occasionally, as in tumour cells, Feulgen positive nucleoli are encountered (Koller, 1943). Such properties, however, give no indication as to the chemical make up of the nucleolus.

ULTRA-VIOLET WORK. The pioneer work in this important field was that of Caspersen and his school (1936, 1939, 1940, 1941) and it is on this work that most of the current views on nucleolar structure are based. The basis of the method is the very strong absorption of nucleic acids at 260m $\mu$ . This characteristic and pronounced maximum allows, with suitable apparatus, the accurate localisation of nucleic acids within the cell. Unfortunately such absorption data do not allow any distinction to be drawn between ribose and deoxyribose nucleic acids, so any differentiation between these two acids must depend on some other technique. For this purpose Caspersen has utilised the Feulgen reaction which he claims (1944) to



localise the position of deoxyribose nucleic acid accurately.

Casperson's technique for the histochemical detection of nucleic acids may therefore be summarised thus; nucleic acids are located in the cell by their characteristic absorption at 260m $\mu$ . after which deoxyribose nucleic acid is demonstrated by means of a positive Feulgen reaction. Any body being Feulgen negative, although having an absorption maximum at 260m $\mu$ , is taken as containing only ribose nucleic acid. On the basis of such results Caspersson and Schultz (1940) claimed that the nucleolus in the eggs of PSALMECHINUS, the root tip cells of SPINACIA, and DROSOPHILA salivary glands contained ribose nucleic acid. From the shape of the absorption curves obtained in the study of nucleoli, Caspersson (1940) claimed that the organelle also contained both histones and higher proteins 'of the albumin type'.

The above technique, however, is open to criticism on several grounds. Firstly there is the very deleterious effect of ultra-violet radiation on the cell. Ris and Mirsky (1949) have shown that even very short exposure to such radiation radically alters the appearance of the cell nucleus so that on this ground alone Caspersson's claims are open to criticism, - as is also his use of 45% acetic acid as a fixative (Caspersson and Schultz, 1940). A much more important criticism than the above, however

is his interpretation of the Feulgen results. Stedman and Stedman (1949) have shown that the Feulgen technique can hardly be expected to locate deoxyribose nucleic acid within the nucleus as under the experimental conditions used the histone is quantitatively removed, along with some 30% of the nucleic acid phosphorus. This being the case, it is extremely obvious that the Feulgen technique can in no way be regarded as showing the distribution of deoxyribose nucleic acid but simply as a particular case of staining with a basic dye (Stedman and Stedman, 1943) so that the nucleolus, as an acidophilic organelle, could not be expected to stain under such conditions.

It would appear, therefore, that Caspersen's claims to have shown the presence of ribose nucleic acid in the nucleus are based on a faulty interpretation of the data available - the only justifiable conclusion being that the nucleus<sup>ol</sup> contains nucleic acid. No conclusions regarding the nature of this nucleic acid are permissible.

Caspersen's claims that histones are present in the nucleolus are also open to criticism. Caspersen (1940) stated that it was possible to differentiate between a 'histone type' and a 'albumin type' of protein by differences in their ultra-violet absorption spectra due to the larger amounts of basic amino acids present in the former. Mirsky and Pollister (1942) have, on the other hand, been unable to show any difference in the absorption spectra of

several different histones and of albumin, when the former are nucleic acid free. Mirsky (1943) has stated that it is very doubtful if Caspersen's 'histone type absorption' is due to histone, but is more probably an artefact due to the latter's use of histones containing large amounts of nucleic acid. Once again it seems that Caspersen's claims to have shown the presence of histone in the nucleolus are not justified.

Some other evidence is, however, available that would appear to support the view that the nucleolus contains ribose nucleic acid. Davidson (1946, 1947) claimed that the ultra-violet absorption of the nucleolus, measured photographically, diminished after ribonuclease treatment but that only the central parts of the nucleolus were attacked, the peripheral regions being unaltered.

In conclusion, therefore, it may be said that from the ultra-violet absorption data available the nucleolus appears to contain nucleic acid and that this may be of the ribose type, although no definite evidence is presented.

STAINING METHODS. In this field the majority of the work is due to Brachet's (1940) development of the Unna-Pappenheim technique. Brachet showed that with this mixture of pyronine and methyl green, both basic dyes, the nucleolus stained specifically red with the pyronine. This red staining no longer occurred after treatment with ribonuclease, which fact was



interpreted as showing the presence of ribose nucleic acid in the nucleolus.

The use of enzymes in a histochemical test is a procedure open to grave criticism, as the exact conditions of the experiment can never be determined. The difficulty in investigating such experiments is well illustrated by the work of Catchside and Holmes (1947) who obtained similar results by using either deoxyribonuclease or egg albumin. Brachet himself used a very impure preparation of ribonuclease but showed (1942) that identical results could be obtained using Kunitz's crystalline ribonuclease (1940) - which, incidentally, still contains traces of proteolytic activity.

The interpretation by Brachet of the fact that ribonuclease removed the pyronine staining of the nucleolus is not the only one possible. Loring (1941) showed that ribonuclease inactivated Tobacco Mosaic Virus and that the inactivation was not due to the hydrolysis of the ribose nucleic acid but to complex formation between the basic enzyme and the acidic virus. Loring pointed out that the inactivation was in no way due to the enzymic properties of the ribonuclease but was simply another example of complex formation between an acidic virus and a basic protein, such as had previously been shown to occur between Bushy Stunt Virus and clupeine (Bawden, 1938) with the resulting inactivation of the virus. It



seemed quite possible, therefore, that an analogous effect could take place in Brachet's histochemical test, - the basic ribonuclease could, by combining with the nucleic acid present in the nucle<sup>ol</sup>us, prevent it from taking up the pyronine from the stain without, however, destroying the nucleic acid, be it ribose or deoxyribose nucleic acid.

The following experiments show that the above explanation is very probable. Rat liver was fixed in Carnoy for 10 minutes, dehydrated and sectioned in paraffin at 10 $\mu$  in the usual way. After various treatments, to be described below, the sections were stained with methyl-green-pyronine (Baker, 1942) for 30 minutes, differentiated for 15 seconds in ethanol and mounted in balsam. Sections were treated as follows-

- A. Incubated for 30mins. in water at 65°C.
- B. Incubated for 30mins. in Ribonuclease at 65°C.
- C. Incubated for 30mins. in inactivated Ribonuclease at 65°C.
- D. Incubated for 30mins. in histone solution at 65°C.
- E. Incubated 30mins. in protamine solution at 65°C.

The ribonuclease was a preparation according to Kunitz (1940) used in a concentration of 1.5mg./ml. The enzyme was inactivated (C.) by boiling for 30 minutes at pH 11, and the histone and protamine were specimens prepared from cod and herring sperm respectively, used in a concentration of 1.5mg./ml. The pH of all these solutions was adjusted to 6.5 - 6.8 before use.

After the above treatment slide A, the control, showed little difference from the untreated slide-the cytoplasm stained red and the nucleolus a purple-red, not the pure red described by Brachet (1942). In all the other slides the staining of the cytoplasm was very much reduced and the nucleoli stained only very faintly indeed.

A similar picture was obtained when mouse liver was smeared directly onto microscope slides, fixed in Carnoy and treated as above before staining with pyronine alone. Again ribonuclease, inactive ribonuclease, histone and protamine were all equally effective in removing the pyronine staining from the nucleolus.

These results would appear to show that Brachet's ribonuclease test for ribose nucleic acid is of little significance and can give no reliable indication as to the distribution of that substance in the cell. It must be noted, however, that in accepting the above explanation there is no evidence to suggest that the nucleolus does not contain ribose nucleic acid. All that may safely be said is that Brachet's interpretation of the results is open to criticism and the question of the nature of the nucleic acid in the nucleolus still left unanswered.

Davidson (1947b) has partially confirmed Brachet's claims. Using ribonuclease he showed that the pyronine staining material was removed from the central portion of the nucleolus. However, using

toluidine blue staining, Davidson and Waymouth (1944d) had previously shown that ribonuclease had no effect on the staining of the nucleolus. The significance of these conflicting results is obscure.

The only other staining technique of importance in the study of the nucleolus is the use of Light Green as a counterstain after the normal Feulgen technique (Gates, 1942; Seshachar, 1942). Light Green stains the nucleolus under such conditions after mordanting for a time with 5%  $\text{Na}_2\text{CO}_3$ , which presumably removes nucleic acid (c.f. Delaporte, 1939). This is claimed to confirm the presence of histone in the nucleolus. As already pointed out, however, histone is quantitatively removed from the nucleus during the acid hydrolysis prior to the Feulgen staining. It is therefore obvious that whatever be the Light Green staining material of the nucleolus it is not histone. This conclusion was borne out by the work of Kaufmann (1947) who showed that pepsin removed the Fast Green staining material from the nucleolus. This Kaufmann claimed to indicate the presence of non-histone protein, although pepsin is well known to digest histone (Kossel, 1928). Kaufmann pointed out the similarity of the distribution of this green staining substance <sup>in the nucleolus</sup> and the chromosomes and that of a tryptophane containing protein, as shown by histochemical methods. Histones are ~~not~~ <sup>not</sup> known <sup>not</sup> to contain tryptophane, therefore, if the nucleolus contains tryptophane, proteins other than histone must be present therein.



The above are almost the only facts available concerning the histochemistry of the nucleolus apart from the work of Gardner (1934) and Gersch (1940) which showed, by micro-incineration techniques, the presence of phosphorus in the nucleolus. The latter worker also suggested that the nucleolus contained phosphoproteins, on the basis of solubility tests.

FUNCTION OF THE NUCLEOLUS. Regarding this point nothing definite is known. Since 1882 the nucleolus has been regarded by many workers as a source or reservoir of chromatin (Flemming, 1882), while others have developed the thesis of Heidenhain (1909) that the nucleolus is an accumulation of the excretory products of the nucleus which are rejected during cell division. This last view has been developed in a modified form by Gates (1942) who has postulated that the nucleolus receives material from the chromosomes at the telophase of the mitotic cycle, and that this material is transferred to the cytoplasm at the following prophase.

Finally, there is the theory of Caspersen already mentioned in the introduction. Caspersen looks upon the nucleolus as an accumulation of the products of heterochromatin which control in some way the synthesis of cytoplasmic ribose nucleoproteins and protein in general.

It is quite obvious, however, that little



more is known of the physiological function of the nucleolus than was known in 1898 when Montgomery published his famous review of the subject.

SIGNIFICANCE OF THE PRESENT INVESTIGATION. Unfortunately, the work reported in the previous sections has done ~~very~~ little to help to clear up the very unsatisfactory state of present day knowledge of the structure and physiology of the nucleolus.

The results would indicate that in normal nuclei there is present ample ribose nucleic acid to allow the nucleolus to contain ribose nucleoproteins. They do not, however, conclusively prove this to be the case. There is definitely a direct relationship between the ribose nucleic acid content of isolated nuclei and the prominence of the nuclear<sup>d</sup> structure therein. The same relationship also exists between the nuclear ribose nucleic acid and the content of the latter substance in the cytoplasm as shown by the figures of Brachet (1942) and of Davidson (1947a). It is thus necessary to assess the validity of the reported ribose nucleic acid content of the nucleus. This has already been discussed to some extent and the conclusion seems inescapable that the values are approximately correct, although they are no doubt maximal due to the presence of non-nucleic acid furfural yielding substances and to small amounts of cytoplasmic material, the latter factor probably being of significance only in the

liver nuclei. These above effects, however, are so small as to have little influence on the magnitude of the reported values and it therefore appears quite justifiable to make the statement that the ribose nucleic acid content of a nucleus is directly proportional to the prominence of the corresponding nucleolar system.

The same statement holds good in the case of regenerating rat nuclei, in which the nuclear ribose nucleic acid is considerably higher than normal. As demonstrated by Novikoff and Potter (1947) the cytoplasmic ribose nucleic acid is also higher in such livers so that once again the danger of contamination is present.

The available evidence, therefore, does not contradict the view that the nucleolus contains ribose nucleic acid, but it in no way shows it to be the case. It is difficult to avoid drawing the conclusion already mentioned, however; namely, the relationship between the prominence of the nucleolar system and the amount of ribose nucleic acid in the cell nucleus.

In the case of fowl erythrocyte nuclei, and to a lesser extent of fish sperm heads, where the small amount of ribose nucleic acid may well be due to contamination, there is, however, no obvious nucleolus and yet there is, at least in the case of the erythrocyte nuclei, a significant amount of ribose

nucleic acid. This immediately opens up the possibility of extra-nuclear<sup>ol</sup> ribose nucleic acid as has indeed been suggested by Caspers~~on~~ and others, especially as a constituent of the heterochromatin. Kaufman (1948) also claimed the presence of ribose nucleic acid in the chromosomes, basing his claims on the use of ribonuclease along with Flemming's triple stain. He postulated that ribose nucleic acid occurred in the chromosomes in a form undergoing cyclic changes during mitosis which led him to suggest a relationship with the reciprocal changes in the nucleolus. Again Mirsky and Ris (1948) showed the presence of ribose nucleic acid in the so called chromosomes isolated from various tissues. Mirsky's evidence for the chromosome nature of his preparations is, however, very poor. From the analyses published it is difficult to escape the conclusion that they are grossly distorted nuclei. Mirsky quotes lymphocyte 'chromosomes' as having a nucleic acid content of 37% which figure is very close to the nucleic acid content of isolated lymphocyte nuclei reported in Table 4 (35% - 36%). This would appear to be adequate evidence that Mirsky's chromosomes are nothing other than badly damaged nuclei. Be this as it may, however, the possibility of extra-nuclear<sup>ol</sup> ribose nucleic acid cannot be excluded.

At this stage of the development of the chemistry of the cell nucleus, therefore, it would not



appear to be unreasonable to assume that the nucleolus contains ribose nucleic acid, although this may well occur elsewhere in the nucleus. This conclusion must not be considered as more than a working hypothesis, and may need radical changes in the light of later work. The final solution to the problem of nucleolar structure will only be obtained, however, when it is possible to isolate this organelle in quantities permitting analyses. From material such as fish ova this should not be an impossible task, although the relationship of such nucleoli<sup>ol</sup> to normal somatic nucleoli could well be questioned. From the very small amount of ribose nucleic acid present in fish sperm, however, it would appear reasonable to assume that the latter substance can play no important function in the genetic mechanisms of the cell.



SUMMARY.

1. Methods of estimation of ribose nucleic acid are discussed and a reliable method for the estimation of that substance in isolated cell nuclei described.
2. An inhibitory effect of deoxyribose nucleic acid on the production of furfural by acid hydrolysis of ribose nucleic acid is discussed.
3. Methods are given for the isolation of various types of cell nuclei.
4. Results are quoted and discussed for the ribose nucleic acid content of cell nuclei isolated from normal and regenerating tissues.
5. The histochemistry of the nucleolus is discussed in relation to the above results and evidence for the presence of ribose nucleic acid in the nucleolus given.
6. A criticism of Brachet's histochemical test for ribose nucleic acid is given, evidence being produced to suggest that the current interpretation of the above test is faulty.
7. The occurrence of ribose nucleic acid in the nucleolus is postulated.

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